PTO/SB/17 (01-06)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL for FY 2006

☒ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 250.00

Complete if Known	
Application Number	09/773,303
Filing Date	January 31, 2001
First Named Inventor	Larry M. Proctor
Examiner Name	McElwain, Elizabeth F.
Art Unit	1638
Attorney Docket No.	414634

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit Card ☐ Money Order ☐ None ☐ Other (please identify) : _____

☒ Deposit Account Deposit Account Number: 12-0600 Deposit Account Name: LATHROP & GAGE LC

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

☒ Charge fee(s) indicated below ☐ Charge fee(s) indicated below, **except for the filing fee**

☒ Charge any additional fee(s) or underpayments of fee(s) Under 37 CFR 1.16 and 1.17 ☒ Credit any overpayments

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
		Small Entity		Small Entity		Small Entity	
Application Type	Fee ($)	Fee($)	Fee($)	Fee($)	Fee($)	Fee($)	Fees Paid ($)
Utility	300	150	500	250	200	100	____
Design	200	100	100	50	130	65	____
Plant	200	100	300	150	160	80	____
Reissue	300	150	500	250	600	300	____
Provisional	200	100	0	0	0	0	____

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims	Extra Claims	Fee($)	Fee Paid ($)
____ -20 or HP=	____	x ____	= ____

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims	Extra Claims	Fee($)	Fee Paid ($)
____ - 3 or HP=	____	x ____	= ____

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims	
Fee ($)	Fee Paid ($)
____	____

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	Extra Sheets	Number of each additional 50 or fraction thereof	Fee ($)	Fee Paid ($)
____ - 100 =	____	/ 50 = ____ (round up to a whole number) x		= ____

4. OTHER FEE(S)

	Fees Paid ($)
Non-English Specification, $130 fee (no small entity discount)	____
Other (e.g., late filing surcharge) : **Appeal Brief**	250.00

SUBMITTED BY

Signature	Dan Lee	Registration No. (Attorney/Agent)	41,935	Telephone	720 931 3000 x 3021
Name (Print/Type)	David J. Lee			Date	October 23, 2006

PATENT
Attorney Docket No.: 414634
Express Mail Label No.: EV196804146US

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

In re Reissue Application of Proctor

Reissue Application No. 09/773,303 — Group Art Unit: 1638

Filed: January 31, 2001 — Examiner: McElwain, Elizabeth F.

For: U.S. Patent No. 5,894,079 — Confirmation No. 6243

In re Proctor Reexamination Proceeding

Control No. 90/005,892

Filed: December 20, 2000

For: U.S. Patent No. 5,894,079

Title: FIELD BEAN CULTIVAR NAMED ENOLA

Mail Stop Appeal Brief – Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

October 23, 2006

APPEAL BRIEF

Sir/Madam:

The present matter is a consolidated reissue/*ex parte* reexamination proceeding being conducted in accordance with 37 C.F.R. §1.565(d).

In accord with 37 C.F.R. §41.37, and fully responsive to the office action of December 21, 2005, Appellants hereby file their appeal brief in support of their appeal in the above-identified matters. A notice of appeal, with the appropriate fee of $250 as required by 37 C.F.R. §§41.31(a) and 41.20(b)(1), was filed on March 21, 2006. The $250 fee for this appeal brief, as required by 37 C.F.R. §41.20(b)(2), is filed herewith. This appeal brief is timely filed within seven months of the mailing of the notice of appeal, with a five-month extension of time. An additional copy of this appeal brief is being submitted on this date, under separate cover, to the Commissioner for Patents, for inclusion in the reexamination proceeding.

10/25/2006 WASFAW1 00000027 1E0600 09773303
01 FC:2402 250.00 DA

10/25/2006 WASFAW1 00000027 09773303
01 FC:2402 250.00 DA

(1) Real party in interest.

The real party in interest for this appeal is Pod-Ners L.L.C., a limited liability corporation established under the laws of the State of Colorado and having a principal place of business at 2611 Highway 348, Delta, CO 81416, U.S.A. The manager of Pod-Ners L.L.C. is Larry M. Proctor. Evidence of assignment of U.S. Patent No. 5,894,079 to Pod-Ners L.L.C. was recorded on May 4, 1999, and may be found at reel/frame 009742/0430.

(2) Related appeals and interferences.

Pod-Ners L.L.C. *v.* Tutuli Produce Corp. *et al.* is an action for infringement of the patent involved in these consolidated proceedings pending in the United States District Court for the Central District of California (Civil Action No. 99-10172-CBM (Mcx)). Defendents have alleged certain counterclaims. The action was stayed on August 20, 2001 pending resolution of these consolidated proceedings and the decision of the United States Supreme Court appeal in J.E.M. AG Supply, Inc. et al. *v.* Pioneer Hi-Bred International, Inc. (No. 99-1996, deciding in favor of the patent and like patents on December 10, 2001).

Pod-Ners v. Northern Feed & Bean *et al.* was an infringement action filed in the United States District Court for the District of Colorado (Civil Action No. 01-CV-2310) on November 30, 2001 (now settled). The suit was for infringement of Pod-Ners Plant Variety Protection Certificate. Certain counterclaims were alleged by defendents.

Pod-Ners v. Ahlberg *et al.* was an infringement and breach of contract action filed in the United States District Court for the District of Colorado (Civil Action No. 05-cv-01866 PSF-MEH) on September 26, 2005 (now settled). The gravamen of the complaint was that defendants unlawfully kept in their possession a crop of Enola beans that had been raised from seed provided them by an affiliate of Pod-Ners. Defendant's understanding with the affiliate was that the beans would be turned over to the affiliate, sold by the affiliate, and the proceeds of the sale returned to the grower.

(3) Status of claims.

Claims 1-53 and 56-64 are pending in the consolidated proceedings. Claims 16-50, 53 and 65-71 are withdrawn from consideration. Claims 1-15, 51, 52 and 56-64 stand rejected.

Specifically, claims 1-15, 51, 52 and 56-64 stand rejected under 35 U.S.C. §112, first paragraph for failing to comply with the written description and enablement requirements; claims 1-7, 57 and 59-64 stand rejected under 35 U.S.C. §112, second paragraph; and claims 1-15, 51, 52 and 56-64 stand rejected under 35 U.S.C. §102(b) as being anticipated by, or in the alternative, under 35 U.S.C. §103(a), as obvious over any of CIAT G13 094, G02 400, G22 215, G22 227, G22 230, G11 891 or Kaplan (Guitarreri Cave, p. 146, 1980), Hernandez-Xolocotzi et al. (Seminar Series 2E, CIAT, p. 253-258, 1973), Voysest (Varieties of Beans in Latin America, CIAT, p. 47-50, 1983), Gepts (The Genetic Resources of Phaseolus Beans, p. 602, 1988), and Azufrado Peruano 87 (Secretaria de Agricultura Y Recursos Hidraulicos; Solicitud de Inscripcion en el Registro Nacional de Variedades de Plantas, Registration No. FRI-150288-042, September 25, 1987).

Appellant appeals claims 1-15, 51, 52 and 56-64 on all grounds of rejection.

(4) Status of amendments.

An *ex parte* reexamination was filed December 20, 2000. A reissue application was filed January 31, 2001. The reexamination and reissue proceedings were consolidated June 13, 2001. A first office action was mailed September 25, 2002. A personal interview was conducted November 19, 2002. On March 25, 2003, a response to the pending office action was filed and entered. On December 2, 2003, a second office action was mailed, and on June 2, 2004, a response was filed and entered. On April 14, 2005, a final rejection was mailed, and on October 14, 2005, a request for continued examination was filed and entered. On December 21, 2005, a final rejection was mailed, prompting a notice of appeal, filed on March 21, 2006.

Claims 1-15, 51, 52 and 56-64 are currently pending, of which claims 1-6, 8 and 11-15 are original (without claim amendment during the consolidated proceedings). Claims 7, 9 and 10 were amended and claims 51, 52 and 56-64 were added in the response of June 2, 2004. Claim 57 was subsequently amended in the RCE of October 14, 2005.

(5) Summary of claimed subject matter.

Claim 1 of U.S. Patent No. 5,894,079 discloses a *Phaseolus vulgaris* field bean seed designated Enola as deposited with the American Type Culture Collection under accession number 209549. A seed deposit was made to the American Type Culture Collection (ATCC) under accession number 209549 on December 11, 1997 (col. 6, lines 9-19).

Claims 5-7 relate to methods of producing a field bean plant by crossing a first parent field bean plant with a second parent field bean plant, wherein the first parent field bean plant, the second parent field bean plant or both are Enola. Methods of producing the Enola field bean plant, for example, by selfing, backcrossing, hybrid breeding, crossing to populations utilizing pollen, protoplasts, plant calli, etc. are disclosed, for example at col. 4, lines 38-54 and Example 2.

Claim 8 recites a field bean variety of *Phaseolus vulgaris* that produces seed having a seed coat that is yellow in color, wherein the yellow color is from about 7.5 Y 8.5/4 to about 7.5 Y 8.5/6 in the *Munsell Book of Color* when viewed in natural light. Claim 13 recites seed from a field bean variety of *Phaseolus vulgaris* that is yellow in color, wherein the yellow color is from about 7.5 Y 8.5/4 to about 7.5 Y 8.5/6 in the *Munsell Book of Color*. Column 1, lines 47-49; column 5, lines 32-36; column 3, lines 32-34 and figures 1 and 2 describe/show the yellow seed.

Claims 9, 10, 14, 15, 52, 60, 61, 63 and 64 recite a field bean variety having a hilar ring, the hilar ring has a color of from about 2.5 Y 9/4 to about 2.5 Y 9/6 in the *Munsell Book of Color* when viewed in natural light. Column 3, lines 39-41 and 44-47, column 5, lines 36-38 and figures 1 and 2 describe/show the hilar ring.

Claim 51 recites seed from a field bean variety of *Phaseolus vulgaris* comprising a seed coat and a hilar ring wherein the seed coat color is about 7.5 Y 8.5/4 to about 7.5 Y 8.5/6 in the *Munsell Book of Color* when viewed in natural light.

The seed being stably reproducible to provide additional seed having the hilar ring and the seed coat color, the seed being produced by a process that includes isolating a population of seed by selection of the hilar ring and the seed coat color from seed products of a segregating population of plants.

Column 1, lines 47-49, column 5, lines 32-36, column 3, lines 32-34 and figures 1 and 2 describe/show the yellow seed. Selection of plants exhibiting favorable characteristics using plant breeder selection criteria (e.g., uniform leaf size, good adherence of pods to branches, resistance to pod shattering, good yield, etc), and reproduction of the plants in subsequent seasons is disclosed at col. 2, line 63 through col. 3, line 29 and col. 4, lines 30-54.

Claim 59 recites seed from a field bean variety of *Phaseolus vulgaris* having a seed coat that is yellow in color, wherein the yellow color plotted as a distribution in a population of the seed of sufficient number for purposes of ATCC deposit has a peak occurrence ranging from about 7.5 Y 8.5/4 to about 7.5 Y 8.5/6 in the *Munsell Book of Color* when viewed in natural light. Column 1, lines 47-49, column 5, lines 32-36, column 3, lines 32-34 and figures 1 and 2 describe/show the yellow seed. A seed deposit was made to the American Type Culture Collection (ATCC) under accession number 209549 on December 11, 1997 (col. 6, lines 9-19).

Claim 62 recites seed from a field bean variety of *Phaseolus vulgaris* having germplasm for expressing a seed coat that is yellow in color as evidenced by a substantially uniform yellow color of the seed coat, wherein the substantially uniform yellow color plotted as a distribution in a population of the seed of sufficient number for purposes of ATCC deposit has a peak occurrence ranging from about 7.5 Y 8.5/4 to about 7.5 Y 8.5/6 in the *Munsell Book of Color* when viewed in natural light. Column 1, lines 47-49, column 5, lines 32-36, column 3, lines 32-34 and figures 1 and 2 describe/show the yellow seed. A seed deposit was made to the American Type Culture Collection (ATCC) under accession number 209549 on December 11, 1997 (col. 6, lines 9-19).

(6) <u>Grounds for rejection to be reviewed on appeal</u>.

A. Whether claims 59-64 are unpatentable under 35 U.S.C. §112, first paragraph, for lack of written description.

B. Whether claims 1-15, 51, 52 and 56-64 are unpatentable under 35 U.S.C. §112, first paragraph, for lack of written description.

C. Whether claims 1-15, 51, 52 and 56-64 are unpatentable under 35 U.S.C. §112, first paragraph, for lack of enablement.

D. Whether claims 1-7, 57 and 59-64 are unpatentable under 35 U.S.C. §112, second paragraph.

E. Whether claims 59-64 are unpatentable under 35 U.S.C. §112, second paragraph for claim language relating to 'the yellow color plotted as a distribution in the population of the seed of sufficient number for purposes of ATCC deposit having a peak occurrence'.

F. Whether claims 1-15, 51, 52 and 56-64 are unpatentable under 35 U.S.C. §102(b) as anticipated by, or in the alternative under 35 U.S.C. §103(a) as obvious over, any of CIAT G13 094, G02 400, G22 215, G22 227, G22 230, G11 891 or Kaplan (Guitarreri Cave, p. 146, 1980), Hernandez-Xolocotzi et al. (Seminar Series 2E, CIAT, p. 253-258, 1973), Voysest (Varieties of Beans in Latin America, CIAT, p. 47-50, 1983), Gepts (The Genetic Resources of Phaseolus Beans, p. 602, 1988), and Azufrado Peruano 87 (Secretaria de Agricultura Y Recursos Hidraulicos; Solicitud de Inscripcion en el Registro Nacional de Variedades de Plantas, Registration No. FRI-150288-042, September 25, 1987).

(7) Arguments.

Patentability under 35 U.S.C. §112, first paragraph, written description, claims 59-64.

The Examiner rejects language in claims 59 and 62, and claims dependent thereon, related to "yellow color plotted as a distribution in a population of the seed of sufficient number for purposes of ATCC deposit [having] a peak occurrence ranging" from one color to another color in the *Munsell Book of Color*. The Examiner states "these phrases were not present in the specification as originally filed, and the subject matter now claimed was not described in the specification in such a way as to

reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention."

There is no requirement that the phrases be recited *ipsis verbis* in the description. See, e.g. *Vas-Cath,* 8935 F.2d at 1563, 19 USPQ2d at 1116; *Martin v. Johnson,* 454 F.2d 746, 751,172 USPQ 391, 395 (CCPA 1972). One skilled in the relevant art would understand that a distribution of color in a population of seed is an inherent trait, and it has been determined that a deposit may be claimed according to the description of properties that are inherent to the deposit, even if those properties are not fully disclosed *in haec verba* in the original specification. *In re Nathan*, 328 F.2d 1005, 1008-1009, 140 USPQ 601, 604 (CCPA 1964) (the court holding that a later-added limitation to the claims of the compound's alpha orientation was "an inherent characteristic" of the claimed subject matter to reverse a new matter rejection); *see Kennecott Corp. v. Kyocera Int'l, Inc.*, 835 F.2d 1419, 1421, (Fed. Cir. 1987) (holding that the disclosure in a subsequent patent application of an inherent property of a product does not deprive that product of the benefit of an earlier filing date because the addition is not new matter).

Distributions of phenotypes are discussed, for example, at the website http://www.mun/ca/biology/scarr/4241F_Quantitative_Genetics.html (July 18, 2005 printout, Evidence Appendix). There are "no clear cut borders for quantitative phenotypes, [we must] deal with a range of phenotypes. For this reason analysis involves measures of central tendency and dispersion." Phenotypic variance may be due to genetic and/or environmental variance such that a central tendency (mean, median or mode) may be identified and used to describe the phenotype. The natural distribution of color in the seed coat of Enola with a peak (central tendency) in the recited range is shown in charts 15 and 31 of the Declaration of Mr. Gil Waibel, a seed expert who studied and reported on this issue (Relevant sections of the Gil Waibel Declaration are included in the Evidence Appendix. The entire Declaration appears in the case file). It is appropriate that claims 59-64 describe inherent properties of the ATCC deposit.

In response to these arguments, the Examiner states that, "*In re Nathan* relates to a chemical compound, which is not analogous to the present claims drawn to bean seeds." (Office Action dated December 21, 2005, p. 4). We note, however, that

Kennecott Corp. v. Kyocera Int'l, Inc. relates to ceramic products, and not to chemical compounds. Thus, the Examiner's narrow interpretation of the case law appears to be inconsistent with precedent. The Examiner further cites MPEP 2163.07(a) and states that "inherency may not be established by possibilities or probabilities." (Office Action dated December 21, 2005, pp. 3-4). It is not, however, merely possible or probable that a sample of seeds within a cultivar will have a distribution of color with a peak occurrence. The peak occurrence is a statistical representation of phenotypic variance, which will occur in the absence of cloning.

In fact, MPEP 2163.07(a) supports the Appellant's position by stating, "By disclosing in a patent application a device that inherently performs a function ***or has a property***...a patent application necessarily discloses that function [or property]..., even though it says nothing explicit concerning it. The application may later be amended to recite the function [or property]...without introducing prohibited new matter. *In re Reynolds,* 443 F.2d 384, 170 USPQ 94 (CCPA 1971); *In re Smythe,* 480 F.2d 1376, 178 USPQ 279 (CCPA 1973)." (emphasis added)

Appellant maintains that claims 59-64 fulfill the requirements of 35 U.S.C. §112, first paragraph, written description.

Patentability under 35 U.S.C. §112, first paragraph, written description, claims 1-15, 51, 52 and 56-64.

Claims 1-15, 51, 52 and 54-58 stand rejected under 35 U.S.C. §112, first paragraph, for lack of written description. The Examiner states that "[t]he claim(s) contains subject matter, which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention..."

We respectfully point out that, "A description as filed is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the examiner to rebut the presumption. See, e.g., *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). The examiner, therefore, must have a reasonable basis to challenge the adequacy of the written description. The examiner has the initial burden of presenting by a preponderance of evidence why a person skilled in the art would not recognize in an applicant's disclosure a description

of the invention defined by the claims. *Wertheim*, 541 F.2d at 263, 191 USPQ at 97." (Guidelines for the Examination of Patent Applications Under the 35 U.S.C. 112, para. 1, "Written Description" Requirement, p. 17).

We do not believe that the Examiner has met the preponderance of evidence standard in stating that the "Patent Owner has not identified what feature or features distinguish the claimed seeds from other known *Phaseolus vulgaris* seeds" (Office Action of April 14, 2005, p. 6; Office Action of December 21, 2005, p. 5) and that "Patent Owner has not set forth what the uniform and stable characteristics are that distinguish the claimed seeds from other known *Phaseolus vulgaris* seeds." (Office Action of April 14, 2005, p. 7; Office Action of December 21, 2005, p. 5). In fact, the Examiner admits that in "Patent Owner's arguments filed October 14, 2005...Patent Owner...[lists] several characteristics as uniform and stable, including the seed coat color and hilar color." (Office Action of December 21, 2005, p. 4).

The Examiner also fails to meet the preponderance of evidence standard in citing a typographical error in the patent specification that reads "The field bean cultivar Enola will *not* be described", instead of "*now* be described", and concluding that "Patent Owner has not described the claimed seeds and plants". This sentence is taken out of context and misconstrued. The next sentence in the paragraph reads, "The terminology used herein *to describe Enola* are those used by the Plant Variety Protection Office, ..." Clearly, Enola is described in the passages of Example 1 and Example 2 that directly follow, as well as by the remainder of the specification, the claims, the figures, and the ATCC deposit.

In the Office Action of December 21, 2005 (p. 5), the Examiner points out that Appellant has not amended the specification to correct the typographical error. We thank the Examiner for bringing this oversight to our attention and, given that we may not make such amendments at this point in the prosecution, we kindly request that an Examiner's amendment be made to change "not" to "now" at col. 4, line 60. Alternatively, Appellant may file a request for a certificate of correction.

The Examiner further states "that Patent Owner has provided evidence that the deposited seeds have many traits that are not stable and uniform." (Office Action of April 14, 2005, p. 8; Office Action of December 21, 2005, p. 5). In support of this view, the Examiner cites the Declaration of Mr. Gil Waibel and Appellant's statement

that, "the seeds deposited with the ATCC are not the seeds of a single genetic entity. Rather the seeds represent a variety of genetic entities, with a range of sizes, shapes and colors, both seed coat and hilar ring."

It is well known that variation within a cultivar can produce a range of sizes, shapes and colors. See, http://www.mun/ca/biology/scarr/4241F_Quantitative_Genetics.html (July 18, 2005 printout forming part of the Evidence Appendix). In accordance with the recognition that uniform and stable traits can best be described as a distribution, the specification of the '079 patent describes "*average* height of the mature plant" (col. 5, line 3), "*average* beak length of the pod" (col. 5, line 29), "number of seeds per pod is *approximately* 3.1", and color ranges from about 7.5 Y 8.5/4 to about 7.5 Y 8.5/6 and from about 2.5 Y 9/4 to about 2.5 Y 9/6 in the *Munsell Book of Color* when viewed in natural light for the seed coat and hilar ring, respectively. In ignoring normal variation among the individuals that make up a cultivar, the Examiner appears to be imposing a stringent test of stability and uniformity that borders on requiring clones. However, the Examiner has stated (Office Action of April 14, 2005, p. 7) that "the Office is not requiring exact [genetic] copies."

The file history shows protracted argument on the subject of genotypes in the context of *University of California v. Eli Lilly and Co.*, 43 USPQ2d 1398 (Fed Cir. 1997), which would "require a precise definition, such as by structure, formula, [or] chemical name, of the claimed subject matter sufficient to distinguish it from other materials" (Office Action of September 25, 2002, p. 14 *et seq.*). The Examiner stated "that phenotypes are the functional expression of the genetic sequences comprised in a plant." (Office Action of April 14, 2005, p. 9). Appellant responded that plant breeders select on the basis of phenotypes, not genetic sequences, and to "require a precise definition, such as by structure, formula, [or] chemical name" of a genetic sequence that forms the basis for a plant phenotype would require a plant breeder or farmer to act in the capacity of a geneticist, molecular biologist, or biochemist. Such a standard of identifying a genetic sequence to determine a basis for a phenotypic trait would be inequitable and would place an undue burden on a plant breeder. The Examiner now states that the Office "...is not requiring that a genetic sequence must be provided to meet the written description requirement." (Office Action of December

21, 2005, p. 6). The Examiner and Appellant appear to be in agreement that possession of the invention may be demonstrated without elucidation of a genetic sequence. As discussed below, Appellant was clearly in possession of the invention at the time of filing application no. 08/749,449, which later issued into the '079 patent.

Appellant has demonstrated possession of the invention based on the deposited material (*Enzo Biochem, Inc. v. Gen-Probe, Inc.*, 63 USPQ2d 1614 (Fed. Cir. 2002)), which may alone be sufficient to meet the written description requirement, and based on an exacting description of uniform and stable traits recited in the description and claims. Further, "possession may be shown by describing an actual reduction to practice of the claimed invention" (Guidelines for the Examination of Patent Applications Under the 35 U.S.C. 112, para. 1, "Written Description" Requirement, p. 9). An actual reduction to practice of the present invention was demonstrated by the ATCC deposit and documented in the drawings/photos of the patent application. (*Vas-Cath,* 935 F.2d at 1565, 19 USPQ2d at 1118 ("drawings alone may provide a 'written description' of an invention as required by Sec. 112")).

"An adequate written description of the invention may be shown by **any description of sufficient, relevant, identifying characteristics** so long as a person skilled in the art would recognize that the inventor had possession of the claimed invention." (Guidelines for the Examination of Patent Applications Under the 35 U.S.C. 112, para. 1, "Written Description" Requirement, p. 9) (emphasis added) citing *Purdue Pharma, L.P. v. Faulding Inc.*, 230 F.3d 1320, 1323, 56 USPQ2d 1481, 1843 (Fed. Cir. 2000). The Examiner cites *University of Rochester v. G.D. Searle & Co., Inc.*, 358 F.3d 926, 69 USPQ2d 1894 (Fed. Cir. 2004), which states that "...the inventor cannot lay claim to that subject matter unless he can provide a description of the compound sufficient to distinguish infringing compounds from non-infringing compounds, or infringing methods from non-infringing methods." Sufficient and relevant description of the Enola cultivar that identifies the stable and uniform characteristics of the plant appears in the '079 patent. The description is exacting enough that a potential infringer would easily know whether or not he was infringing, for example, by comparing the color of a potentially infringing field bean viewed in natural light to the *Munsell Book of Color*.

Appellant respectfully traverses the §112, first paragraph, written description rejection.

Patentability under 35 U.S.C. §112, first paragraph, enablement, claims 1-15, 51, 52 and 56-64.

Claims 1-15, 51, 52 and 56-64 stand rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement.

The Examiner refers to "a heterogeneous mix of seeds in the deposit" and "the deposit of a phenotypically varied population of seeds" (Office Action of April 14, 2005, pp. 11-12; Office Action of December 21, 2005, p. 7). As described above, the Enola cultivar comprises a plurality of individuals that share one or more uniform and stable traits. The heterogeneity of the seeds is on an individual basis – they are not clones. The deposit contains a population of seeds displaying a normal variation in size, shape, color and other traits; the seeds are not phenotypically varied, but they display normal phenotypic variance. See http://www.mun/ca/biology/scarr/4241F_Quantitative_Genetics.html (July 18, 2005 printout forming part of the Evidence Appendix).

The Examiner states that the Declaration of Mr. Gil Waibel describes variation in Enola. Specifically, the Examiner notes that the primary color of seed coat addressed at item # 42 of the declaration asserts that "most" of the seed was in a color range described in the Plant Variety Protection application, which is different from the seed color range claimed and disclosed in the specification (Office Action of April 14, 2005, p.11; Office Action of December 21, 2005, p. 7). However, at point # 42 of the Declaration, Mr. Gil Waibel references charts 15 and 31, which clearly show that the peak occurrence of Enola seed coat color occurs between 7.5 Y 8.5/4 to 7.5 Y 8.5/6, as claimed and recited in the patent specification. In charts 15 and 31, 85% and 80% of the Enola seeds examined showed color in the 7.5 Y 8.5/4 to 7.5 Y 8.5/6 range.

The Examiner states that the "Patent Owner has not provided guidance with regard to how one skilled in the art would select from the heterogeneous seeds that are deposited to identify individuals that would be considered to be Enola yellow beans." (Office Action of December 21, 2005, p. 7). We reiterate that the heterogeneity of the

seeds is on an individual basis – they are not clones. The heterogeneity of the seeds presents as phenotypic variance, which is a well know phenomenon for living matter. Each and every seed on deposit belongs to the Enola cultivar. Therefore, one of skill in the art could easily reproduce the claimed invention, for example, by accessing the ATCC deposit, as any seed on deposit would produce a plant of the claimed cultivar.

The Examiner also states that "the purpose of the deposit of seed is to provide a reproducible means of making a unique plant that cannot be recreated based on a patent disclosure." (Office Action of December 21, 2005, p. 7). The Office appears to be asserting a single legal purpose for making a seed deposit, where there may be other motivating factors, e.g., to satisfy the written description requirement, *vide supra*. In fact, "[i]t has been determined by the court that availability of the biological product via a public depository provided an acceptable means of meeting the **written description and the enablement requirements** of 35 U.S.C. 112, first paragraph." MPEP 2164.06(a) citing *In re Argoudelis,* 434 F.2d 1390, 168 USPQ 99 (CCPA 1970). (emphasis added)

Appellant respectfully traverses the §112, first paragraph, enablement rejection.

Patentability under 35 U.S.C. §112, second paragraph, claims 1-7, 57 and 59-64.

Claims 1-7, 57 and 59-64 stand rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Specifically, the Examiner objects to Appellant's statement that the seeds deposited with the ATCC are not seeds of a single genetic entity. As discussed above, the seeds deposited with the ATCC are of a single cultivar (Enola), which is comprised of a plurality of individuals that have unique genetic profiles – the individuals are not clones. As "the Office is not requiring exact [genetic] copies" (Office Action of April 14, 2005, p. 7), Appellant fails to see the validity of this rejection.

The Examiner also objects to "Patent Owner assert[ing] that they do not agree or disagree that the cultivar contains a uniform genotype." (Office Action of April 14, 2005, p. 14). The Examiner then argues that by making this statement the "Patent Owner has admitted that the metes and bounds of the claimed invention are not clear."

(Office Action of April 14, 2005, p. 14). We respectfully disagree. According to the Merriam-Webster Online Dictionary, a genotype is all or part of the genetic constitution of an individual or group. Patent Owner has already stated that the Enola cultivar does not consist of individuals with identical (uniform) genetic constitutions (i.e., the Enola cultivar is not comprised of cloned plants). The Merriam-Webster Online Dictionary also defines a phenotype as the visible properties of an organism that are produced by the interaction of the genotype and the environment. Since the Enola phenotype is stable and uniform in multiple environments, we must assume that at least part of the genotype (responsible for the visible properties) is uniform.

The Examiner states that because "Patent Owner continues to argue that a uniform genotype only requires that part of the genotype is uniform. It is clear that the specification does not define the full range of seed phenotypes and genotypes that are comprised in the deposit, such that one skilled in the art would be able to determine what materials would infringe the claim." (Office Action of December 21, 2005, pp. 8-9). The claims utilize phenotypic language, which is exacting enough that a potential infringer would know whether or not he was infringing, for example, by comparing the color of a potentially infringing field bean viewed in natural light to the *Munsell Book of Color*. Where the claims define the invention using phenotypic language, we fail to understand the Examiner's point that "the specification does not define the full range of seed...genotypes that are comprised in the deposit."

Appellant respectfully traverses the §112, second paragraph rejection.

Patentability under 35 U.S.C. §112, second paragraph, claims 59-64.

The Examiner deems the phrases "wherein the yellow color plotted as a distribution in the population of the seed of sufficient number for purposes of ATCC deposit has a peak occurrence ranging..." and "as evidenced by a substantially uniform yellow color plotted as a distribution in a population of the seed of sufficient number for purposes of ATCC deposit has a peak occurrence ranging..." to be indefinite because "the phrase[s] [are] not comprehensible. It is not clear what is intended, and it is not clear what yellow color the seed could be." (Office Action of December 21, 2005, p. 9).

Claim 59 recites seed from a field bean variety of *Phaseolus vulgaris* having a

seed coat that is yellow in color, wherein the yellow color plotted as a distribution in a population of the seed of sufficient number for purposes of ATCC deposit has a peak occurrence ranging from about 7.5 Y 8.5/4 to about 7.5 Y 8.5/6 in the *Munsell Book of Color* when viewed in natural light. Recordation of phenotypic observations as a function of frequency is a common tool for plant breeders. One skilled in the art would clearly comprehend that when observations of the yellow seed coat color of Enola are recorded as a function of frequency for 2500 seeds (i.e., a population of the seed of sufficient number for purposes of ATCC deposit) in natural light, phenotypic variance will produce a peak occurrence of seed coat color between about 7.5 Y 8.5/4 to about 7.5 Y 8.5/6 in the *Munsell Book of Color*. In fact, this is precisely the analysis that Mr. Gil Waibel reported in his Declaration (charts 15 and 31), where 80-85% of the sampled Enola seeds had a yellow seed coat color within the claimed range.

Claim 62 recites seed from a field bean variety of *Phaseolus vulgaris* having germplasm for expressing a seed coat that is yellow in color as evidenced by a substantially uniform yellow color of the seed coat, wherein the substantially uniform yellow color plotted as a distribution in a population of the seed of sufficient number for purposes of ATCC deposit has a peak occurrence ranging from about 7.5 Y 8.5/4 to about 7.5 Y 8.5/6 in the *Munsell Book of Color* when viewed in natural light, and benefits from like argument.

Appellant respectfully traverses the §112, second paragraph rejection.

Patentability under 35 U.S.C. §102/103 claims 1-15, 51, 52 and 56-64.

Claims 1-15, 51, 52 and 56-64 stand rejected under 35 U.S.C. §102(b) as anticipated by, or in the alternative, under 35 U.S.C. §103(a) as obvious over CIAT G13 094, G02 400, G22 215, G22 227, G22 230, G11 891, or Kaplan (Guitarrero Cave, p. 146, 1980), or Hernandez-Xolocotzi et al. (Seminar Series 2E, CIAT, p. 253-258, 1973), or Voysest (Varieties of Beans in Latin America, CIAT, p. 47-50, 1983), or Gepts (The Genetic Resources of Phaseolus Beans, p. 602, 1988), or Azufrado Peruano 87 (Secretaria de Agricultura Y Recursos Hidraulicos; Solicitud de Inscripcion en el Registro Nacional de Variedades de Plantas, Registration No. FRI-150288-042, September 25, 1987 at No. 52 and 53, in the IDS filed November 15,

2002).

The Examiner bases this rejection on beans (or bean collections) that neither the Examiner nor the Appellant has ever seen or been able to find (Kaplan, Hernandez, Voysest or Gepts). However, elusive references like these cannot support the Examiner's burden of making out a *prima facie* case of anticipation based on these references.

Examiner attempts to make her elusive references real by blaming Appellant for failing to find them and test them (Office Action of April 14, 2005, p. 20; Office Action of December 21, 2005, p. 11). However, the burden is on the Examiner, not applicant, and the Examiner plainly has not carried her burden. Appellant has honestly and directly tried diligently to find prior art, and has been doing so since the earliest days of what turned out to be the Enola project. The Examiner has been provided with everything that Appellant and his colleagues have been able to find, and they have so sworn honestly, repeatedly and under penalty of perjury. The Examiner apparently believes, without saying so directly, that they have sworn falsely.

The Examiner supports her reliance on elusive references with a variety of arguments, including the argument that "the claimed bean seeds are clearly anticipated, as evidenced by the Polly Proctor Declaration, . . . and further given the subjective nature of color determination under conditions of natural light" (Office Action of April 14, 2005, p.20). The first point is meritless, and the second is a reliance on the very evidence that the Examiner cites to support the first argument -- the Polly Proctor Declaration (Evidence Appendix).

Another elusive reference relied upon by Examiner is Peruano 87 (Office Action of April 14, 2005, p. 20). The Examiner's principal evidence on this subject is testimony of Dr. Pfeiffer (once a co-owner of Pod-Ners) to the effect that the Enola bean *is* Peruano 87 (Pod-Ners' Response to Defendants' Motion to Compel. Portions of the Response are attached in the Evidence Appendix. The entire Response forms part of this case file.).

There are several flaws in this approach. The testimony was given in litigation over Pod-Ners' Plant Variety Protection Certificate. By the time Dr. Pfeiffer gave the testimony, Pod-Ners had been involved in separate litigation with Dr. Pfeiffer over

management of Pod-Ners, the company that owns the patent in issue here. Furthermore, the cited testimony was taken by the defendants in the PVP action, and Appellant did not have an opportunity to cross-examine before the case settled - - although this may not matter. Dr. Pfeiffer vacillated extensively in answering the deposition questions of relevance. This testimony further contradicts the testimony cited by the Examiner:

> Q: What about the Peruano 87? Was that the closest out of – closest to the Enolas out of all of the varieties regardless of geographic location?
>
> A: I can't unequivocally say yes or no to that question because I don't know specifically the origin of the seed.

(Dr. Pfeiffer Depo., p. 287.)

Dr. Pfeiffer also stated that Pimono 78 was disclosed to the PVP Office rather than Peruano 87 because he did not have any Peruano 87 with which to compare to the Enola variety.

> Q: Why is it that you were able to tell the PVP Office that Pimono 78 was the closest?
>
> A: Because we had – originally the Bud originated from Mayocoba, as far as I know. And in the Mexican publication they had listed Pimono 78 as a Mayocoba, and that's the only other thing that we had that we could make any possible comparison to.

Dr. Pfeiffer also testified that he attempted to get some Peruano 87 seed in 1997 to make a color comparison with Enola. However, the seed that he obtained was treated and resulted in distorted and unreliable results. (Dr. Pfeiffer Depo., p. 300-01.)

Dr. Pfeiffer's uncertainty on the parentage was based in part on his experience that seed from Mexico, even allegedly registered seed, may not conform to its labeling.

> Q: So how do you know what it is?
>
> A: Oftentimes you don't. Even if it says it's a variety, you are not even quite sure that's, quote, the variety because I don't think the Mexican government maintains their seed stock from – . . .

(Dr. Pfeiffer Depo., p. 148.)

> Q: What was your concern about comparing it to the parents?
>
> A: Essentially didn't know what the parentage was. The seed that was planted in Delta, Colorado, I still don't know what the parentage of that was specifically, and even if it, again, was a variety that originated in Mexico, my theory on seed out of Mexico is that what you see – what you get and what's actually in the bag may be two totally different things.

(Dr. Pfeiffer Depo., pp. 281-82.)

The Examiner next tries to support her anticipation holding by relying on Polly Proctor's color analyses under the *Munsell Book of Color* (Office Action of April 14, 2005). The analyses are based on nine specific, tangible beans that Appellant was able to find and have tested by Ms. Proctor for the Examiner. As the Examiner admits, none of the readings fall within the scope of the claims. In the words of the patent, none of the nine beans "possesses a unique yellow color matching most closely to 7.5Y 8.5/4 to 7.5Y 8.5/6 in the *Munsell Book of Color* when viewed in natural light" (col.3, lines 31-34; col. 1, line 65 to col. 2, line 4 (includes hilar ring)). Of the 111 readings taken, only one even fell on the 7.5Y page of the *Munsell Book of Color*, and that single point was outside the claims.

The Examiner deals with this flaw in her anticipation position by construing the term "about" in the numerical limitations of the claims. Although the Examiner does not say so explicitly, her claim construction plainly is meant to encompass Munsell color squares that surround the two claimed squares -- 8.5/4 and 8.5/6 on page 7.5Y of the Munsell Book of Color. There is no support for this approach anywhere in the unusually large record in this matter. The Examiner's claim construction is made in a single sentence, and no supporting authority, factual or legal, is cited in support (Office Action of April 14, 2005, p. 18). It is an understatement to say that the Examiner has not carried her burden of making out a *prima facie* case of anticipation.

In his early work with the Enola bean, the Appellant found that the seed coat color consistently fell on the 7.5Y page of the Munsell Book and consistently matched the 8.5/4 and 8.5/6 color sample squares on the 7.5Y page, which were next to one another (L. Proctor Declaration at 3, Evidence Appendix). The match was not always

perfect, however, because the two Munsell squares were two single colors and the beans were not always those exact colors. As the patent puts it: "Enola seed possesses a unique yellow color *matching most closely* to 7.5Y 8.5/4 to 7.5Y 8.5/6 in the Munsell Book of Color when viewed in natural light" (col. 3, lines 31-34; col. 1, line 65 to col. 2, line 4 (includes hilar ring)) (emphasis added). The same general circumstances were observed with other parts of the Enola seed and plant (L. Proctor Declaration at 4).

The word "about" in the patent claims was put there to deal with this issue, which applicant specifically discussed with his patent lawyer (L. Proctor Declaration at 5). Applicant hoped that people would not be able to avoid his patent claims with beans that were not the exact shade of the squares he specified (L. Proctor Declaration at 5).

Applicant did not and does not intend, however, to try to stretch or distort "about" in such a way as to gain claim coverage of a bean matching some Munsell color square other than the specified 8.5/4 and 8.5/6 squares on the 7.5 Y page of the *Munsell Book of Color* (L. Proctor Declaration at 6). It was Mr. Proctor's understanding and intent that beans having, for example, a seed coat color of 7.5Y 8.5/8 or 7.5Y 8/6 would not fall within his claimed range of "about 7.5Y 8.5/4 to about 7.5Y 8.5/6" (L. Proctor Declaration at 6).

Having attempted to make out a case of anticipation based on the work of Ms. Proctor, the Examiner then devotes considerable energy to discrediting Ms. Proctor's work on grounds of subjectivity, incompetence and bias (Office Action of April 14, 2005, p. 18) . This is wasted energy. Accepting *arguendo* the Examiner's rejection of Ms. Proctor, it follows that the Examiner's entire anticipation case is based on five elusive references that the Examiner has never seen and a color analysis that the Examiner considers to be worse than useless.[1] In short, the Examiner has made *no* case whatsoever of anticipation, colorable, *prima facie* or otherwise.

---

[1] Although probably not relevant for present purposes, Ms. Proctor is in fact a skilled colorist, with years of experience in reading bean colors. She used the Munsell test because it was recommended by the Plant Variety Protection Office. When she visited the Munsell laboratories, she took and passed the Farnsworth-Munsell Color test, a highly effective method for measuring any individual's color vision that has been used by the government and industry for over 40 years. The

The Examiner has also cited Bassett et al., ASHS Journals Online (2000) (Evidence Appendix) and the Expert Report of Paul Gepts, Ph.D. and expresses concern that "given the differing results of Bassett and Gepts with regard to identifying the parentage of Enola, ... the genetic makeup of Enola appears to be in question." (Office Action of April 14, 2005, p. 19). We fail to understand the Examiner's point. Even if the parentage of Enola was known, it would still be possible that the complete genome of Enola and/or the parents would not be elucidated. We reiterate that plant breeders select on the basis of phenotypes, not genetic sequences; therefore, it is inapposite that the Examiner considers "the genetic makeup of Enola...to be in question."

The Examiner cites Pallotini et al. (Crop Science 44: 968-977, 2004, Evidence Appendix) and states that "the results of their study indicate that the claimed Enola bean has an identical fingerprint to yellow-seeded beans from Mexico and is most similar to Azufrado Peruano 87" (Office Action of April 14, 2005, p. 19). Respectfully, we also point out that the Pallotini article states, "Calculations of the probability of matching AFLP fingerprints showed that the most likely origin of Enola is by selection within pre-existing Mexican Peruano-type cultivars. This finding is consistent with the history of this genotype as outlined in the Enola patent and Appendix A of the PVP certificate...The uniformity of the AFLP banding pattern suggests that the sample submitted to the ATCC resulted from single seed selection during several generations before submission of the required seed sample to the ATCC." (p. 976). The Pallotini article supports Appellant's position that Enola was produced through a selection process performed on beans, which were most likely of Mexican origin. We believe that Pallotini's conclusion resulted even though the study utilized selective and biased data, as explained below.

The Pallotini study is little more than a reprint of the Expert Report of Paul Gepts, Ph.D., which was addressed by and submitted with the March 25, 2003 Declaration of Laura Conley (Evidence Appendix). The Pallotini/Gepts study selectively analyzed Enola data from a "monomorphic" ATCC deposit, although the authors were clearly aware of at least two haplotypes within the ATCC material that

---

Examiner's discussion of color issues is incorrect in several respects (P.Proctor Dec. at 2-8).

they had in their possession (individuals #1 (Enola 2000-1), #52 (Enola 2000-2) and possibly #56 (Enola 2002) from Table 1 of the Pallotini article/Expert Report of Paul Gepts, Ph.D.(see specifically supporting data of Experiment 1 submitted as Exhibit B of the Laura Conley Declaration)). Further, Figures 2 and 4 of the Pallotini publication/Paul Gepts Report show polymorphism among Enola samples from all sources, e.g., Enola-1 (ATCC-USA), Enola-2001 (NFB-USA), Enola (NFB-USA).

By ignoring polymorphism within the Enola cultivar, Pallotini *et al.* created biased results that favored similarity between Azufrado Peruano 87 and Enola. Even after biasing the results, the authors found only a 30% chance that Enola resulted from selection without hybridization within the Azufrado Peruano 87 cultivar. (Table 3 shows that the probability of selection without crossing from Azufrado Peruano 87 was determined to be 3×10^{-1}.) These results are highly suspect and completely unconvincing. In the Office Action of December 21, 2005 (p. 13), the Examiner states that it is unclear "what the relevance of this [probability discussion] is with regard to the novelty of the claimed Enola bean." The relevance is that the Examiner is relying on a reference that teaches away from the Examiner's position. The Pallotini publication shows that it is more unlikely than likely that Enola resulted from Azufrado Peruano 87 without crossing.

The Examiner appears to misunderstand how Pallotini's results were biased. Pallotini's results were biased toward similarity between Enola and Azufrado Peruano 87 because the researchers selected for one haplotype of Enola, when they were clearly aware of multiple haplotypes. The presence of multiple haplotypes is consistent with Appellant's position that the seeds are not clones – each individual may have a unique genetic profile, so long as it exhibits the claimed phenotypic trait(s). Appellant is not admitting, or even suggesting, that the seeds of the Enola ATCC deposit are anticipated by Azufrado Peruano 87. There is no prior art admission.

Rebuttal of cited art

Kaplan (Guitarrero Cave, p. 146, 1980) (Evidence Appendix) discloses a yellow *Phaseolus vulgaris* variety with median seed dimensions of 1.1 (length) x 0.79 (width) x 0.69 (thickness) cm, 4 seeds per pod and spherical pod morphology. Enola

has an average seed length of 1.27 cm (based on a sample from Figure 1 of the '079 patent – scaled relative to a U.S. nickel), 3.1 seeds per pod and a pear shape cross section (col. 5, lines 26-31). Enola does not fit the characteristics of the variety disclosed by Kaplan.

Hernandez-Xolocotzi (Seminar Series 2E, CIAT, p. 253-258, 1973) (Evidence Appendix) discloses in Table 2 approximately 200 yellow *Phaseolus vulgaris* of various diameters as measured in millimeters. The values reported appear to be far too small for normal beans and no specific varieties are listed. We are unable to address a rejection based on such a vague publication. Further clarification or withdraw of the rejection is requested.

Voysest (Varieties of Beans in Latin America, CIAT, p. 47-50, 1983) (Evidence Appendix) discloses Canario, Azufrado and Azufrado Peruano varieties. Voysest lists all of the varieties as "medium size". According to his definition (p. 5) medium is "between 25 and 40 grams/100 seeds" and large is "from 40 grams/100 seeds". The '079 patent lists Enola as 43 grams per 100 seeds (col. 5, line 42) and the Declaration of Gil Waibel found the Enola seeds to be between 47-58 g/100 seeds, thus falling outside of the medium classification of the Canario, Azufrado and Azufrado Peruano varieties.

In response to the arguments relating to Kaplan, Hernandez-Xolocotzi and Voysest, the Examiner states that "it is not shown that these differences are statistically significant." (Office Action of December 21, 2005, p. 13). In so stating, the Examiner appears to be imposing an artificially high evidentiary standard, and weighing the evidence against the *prima facie* case itself rather than against the facts on which the conclusion of a *prima facie* case was reached; see *In re Eli Lilly,* 902 F.2d 943, 14 USPQ2d 1741 (Fed. Cir. 1990); *In re Piasecki,* 745 F.2d 1468, 1472, 223 USPQ 785, 788 (Fed. Cir. 1984). In the present case, Kaplan, Hernandez-Xolocotzi and Voysest vaguely disclose yellow *Phaseolus vulgaris* by providing limited descriptions of such seeds. Appellant has presented rebuttal evidence that these limited descriptions do not match those of Enola. "The ultimate determination of patentability must be based on consideration of the entire record, by a preponderance of evidence..." *In re Oetiker,* 977 F.2d 1443, 24 USPQ2d 1443 (Fed. Cir. 1992). Where a weak or nonexistant *prima facie* case was presented and

reasonable rebuttal evidence has been provided, Appellant has shown by a preponderance of the evidence that the cited references do not anticipate Enola.

Appellant respectfully traverses the §102/§103 rejection.

Requirement for Information under 37 C.F.R. §1.105.

The Examiner is continuing to require Appellant to provide any information available regarding the sale within the ambit of Appellant's control or that of a third party or the public use in the United States of the filed bean seeds originally obtained in Mexico. Regarding sales within the Appellant's ambit, Appellant developed his invention in secrecy and filed his application less than one year prior to his first public disclosure of his invention. Regarding sales by third parties and public use, Appellant is unaware of any such activities in the United States at the time of the original purchase and/or prior to the filing date of the instant patent. We traverse this continued request: "Any reply that states that the information required to be submitted is unknown and/or is not readily available to the party or parties from which it was requested will be accepted as a complete reply." (37 CFR §1.105(a)(3)).

The Examiner also requests clarification as to where the following statement was made:

> "Applicant planted the collection of yellow beans in 1991, and pursued a program of selective breeding for three generations. The invention was complete in 1993. For purposes of improving stability, applicant continued to selectively breed the invention through perhaps 1997. The patent states that the Mexican beans were acquired in 1994 and the breeding program started then. This is incorrect. It is an error. The same error was made in applicant's application for a Plant Variety Protection Act Certificate..."

The Board is directed to the Office Action Response filed March 25, 2003, p. 15. The Examiner further states that, "if Patent Owner is stating that the date for beginning development of Enola bean of 1994 and the manner in which it was derived is in error in both the patent and in the application for Plant Variety Protection Act Certificate, then information with regard to the circumstances surrounding the error must be

provided." Appellant has stated that the date for beginning development of Enola in the patent and PVP Certificate are incorrect; however, the manner in which it was derived is correct.

(8) Claims Appendix.

Appellant encloses a copy of the claims involved in this appeal as an appendix hereto.

(9) Evidence Appendix.

Appellant encloses a copy of the evidence involved in this appeal as an appendix hereto.

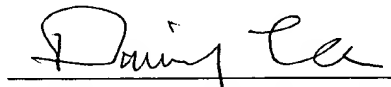
(10) Related Proceedings Appendix.

To Appellant's knowledge, there are no decisions rendered by a court or the Board for submission with this appeal.

Conclusions

Appellant respectfully submits that claims 1-15, 51, 52 and 56-64 satisfy the written description and enablement criteria, particularly point out and distinctly claim the subject matter that Appellant regards as the invention, and patentably distinguish over the art of record. Authorization to charge fees associated with a five-month extension of time and the cost for this appeal brief is submitted herewith. If any additional fee is deemed necessary, the Commissioner is hereby authorized to charge such fee to Deposit Account No. 12-0600.

Respectfully submitted,

By: 

David J. Lee, Reg. No. 41,935
LATHROP & GAGE L.C.
4845 Pearl East Circle, Suite 300
Boulder, CO 80301
Telephone: (720) 931-3021
Facsimile: (720) 931-3001

CLAIM APPENDIX TO APPEAL BRIEF

1. (Original) A *Phaseolus vulgaris* field bean seed designated Enola as deposited with the American Type Culture Collection under accession number 209549.

2. (Original) A field bean plant produced by growing the seed of claim 1.

3. (Original) Pollen of the plant of claim 2.

4. (Original) A field bean plant having all the physiological and morphological characteristics of the field bean plant of claim 2.

5. (Original) A method of producing a field bean plant comprising crossing a first parent field bean plant with a second parent field bean plant, wherein the first field bean plant is the field bean plant of claim 2.

6. (Original) A method of producing a field bean plant comprising crossing a first parent field bean plant with a second parent field bean plant, wherein the second field bean plant is the field bean plant of claim 2.

7. (Previously presented) A method of producing a field bean plant comprising crossing a first parent field bean plant with a second parent field bean plant, wherein the first field bean plant and the second field bean plant is the field bean plant of claim 2.

8. (Original) A field bean variety of *Phaseolus vulgaris* that produces seed having a seed coat that is yellow in color, wherein the yellow color is from about 7.5 Y 8.5/4 to about 7.5 Y 8.5/6 in the *Munsell Book of Color* when viewed in natural light.

9. (Previously presented) The field bean variety of claim 8 wherein the seed further comprises a hilar ring.

10. (Previously presented) The field bean variety of claim 9 wherein the hilar ring has a color of from about 2.5 Y 9/4 to about 2.5 Y 9/6 in the *Munsell Book of Color* when viewed in natural light.

11. (Original) Propagation material of the *Phaseolus vulgaris* of claim 8.

12. (Original) Pollen of the *Phaseolus vulgaris* of claim 8.

13. (Original) Seed from a field bean variety of *Phaseolus vulgaris* that is completely yellow in color, wherein the yellow color is from about 7.5 Y 8.5/4 to about 7.5 Y 8.5/6 in the *Munsell Book of Color*.

14. (Original) Seed of claim 13 further comprising a hilar ring.

15. (Original) Seed of claim 14 wherein the color of the hilar ring is from about 2.5 Y 9/4 to about 2.5 Y 9/6 in the *Munsell Book of Color* when viewed in natural light.

16. (Withdrawn) A *Phaseolus vulgaris* field bean plant, said plant comprising a wood-like stalk and a plurality of wrinkled, dull, ovate-shaped leaves.

17. (Withdrawn) The plant of claim 16, wherein said plant comprises a plurality of white flowers.

18. (Withdrawn) The plant of claim 16, wherein at least one of said flowers comprises a plurality of white wings.

19. (Withdrawn) The plant of claim 16, wherein at least one of said flowers comprises a white keel.

20. (Withdrawn) The plant of claim 16, wherein said plant comprises a plurality of pods whose positions on said plant are scattered.

21. (Withdrawn) The plant of claim 16, wherein said plant comprises a plurality of flowers and pods, said stalk, leaves, flowers and pods being free from anthocyanin.

22. (Withdrawn) The plant of claim 16 wherein the apex of said leaves are acuminate and the base of said leaves is obtuse.

23. (Withdrawn) The plant of claim 22, wherein the average height of said plant when mature is about 34.9 cm.

24. (Withdrawn) The plant of claim 22, wherein said plant has lodging resistance through maturity and withstands wind and other climatic conditions.

25. (Withdrawn) The plant of claim 22, wherein said plant establishes a long, deep-growing, wood-like taproot, a plurality of wood-like lateral roots, and a plurality of wood-like feeder roots.

26. (Withdrawn) The plant of claim 25, wherein said taproot is larger than at least one of said lateral roots, and at least one of said lateral roots is larger than said feeder roots.

27. (Withdrawn) The plant of claim 25, wherein said taproot averages 1.0 cm +/- in caliper size.

28. (Withdrawn) A pod of a *Phaseolus vulgaris* field bean plant having, at onset, a solid green color pattern, wherein said color is about 5 GY 6/6 in the *Munsell Book of Color* when viewed in natural light.

29. (Withdrawn) The pod of claim 28, wherein said pod has a pear-shaped cross section.

30. (Withdrawn) The pod of claim 29, wherein the curvature of said pod is straight and the orientation of the beak of said pod is straight.

31. (Withdrawn) The pod of claim 30, wherein said pod has slight constrictions.

32. (Withdrawn) A pod of a *Phaseolus vulgaris* field bean plant having, at maturity, a solid tan color pattern, wherein said color is about 5 Y 8.5/6 in the *Munsell Book of Color* when viewed in natural light.

33. (Withdrawn) The pod of claim 32, wherein said pod has a pear-shaped cross section.

34. (Withdrawn) The pod of claim 32, wherein said pod is slightly curved and the orientation of the beak of said pod is variable.

35. (Withdrawn) The pod of claim 32, wherein said pod has slight constrictions.

36. (Withdrawn) The pod of claim 32, wherein the average beak length of said pod is 1.2cm.

37. (Withdrawn) The pod of claim 32, wherein said pod comprises seeds and the number of said seeds per pod is approximately 3.1.

38. (Withdrawn) A *Phaseolus vulgaris* field bean plant, said plant comprising a wood-like stalk, at least one pod, and a plurality of wrinkled, dull, ovate-shaped leaves.

39. (Withdrawn) The plant of claim 38, wherein said pod has, at onset, a solid green color pattern, wherein said color is about 5 GY 6/6 in the *Munsell Book of Color* when viewed in the natural light.

40. (Withdrawn) The plant of claim 39, wherein said pod has a pear-shaped cross section.

41. (Withdrawn) The plant of claim 40, wherein said pod is straight and the orientation of the beak of said pod is straight.

42. (Withdrawn) The plant of claim 41, wherein said pod has slight constrictions.

43. (Withdrawn) The plant of claim 38, wherein said pod has, at maturity, a solid tan color pattern, wherein said color is about 5 Y 8.5/6 in the *Munsell Book of Color* when viewed in natural light.

44. (Withdrawn) The plant of claim 43, wherein said pod has a pear-shaped cross section.

45. (Withdrawn) The plant of claim 43, wherein said pod is slightly curved and the orientation of the beak of said pod is variable.

46. (Withdrawn) The plant of claim 43, wherein said pod has slight constrictions.

47. (Withdrawn) The plant of claim 43, wherein the average beak length of said pod is 1.2cm.

48. (Withdrawn) The plant of claim 43, wherein said pod comprises seeds and the number of said seeds per pod is approximately 3.1.

49. (Withdrawn) A method of harvesting a *Phaseolus vulgaris* field bean plant, said method comprising the following steps:

a. knifing the plant;

b. placing the plant into a windrow;

c. allowing the plant to dry.

50. (Withdrawn) The method of claim 49 wherein said drying step is continued for approximately 5 to 8 days.

51. (Previously presented) Seed from a field bean variety of *Phaseolus vulgaris* comprising a seed coat and a hilar ring wherein the seed coat color is about 7.5 Y 8.5/4 to about 7.5 Y 8.5/6 in the *Munsell Book of Color* when viewed in natural light;

the seed being stably reproducible to provide additional seed having the hilar ring and the seed coat color,

the seed being produced by a process that includes isolating a population of seed by selection of the hilar ring and the seed coat color from seed products of a segregating population of plants.

52. (Previously presented) The seed of claim 51 wherein the hilar ring color is from about 2.5 Y 9/4 to about 2.5 Y 9/6 in the *Munsell Book of Color* when viewed in natural light.

53. (Withdrawn) A seed from a field bean variety of *Phaseolus vulgaris* comprising a seed coat and a hilar ring wherein the hilar ring color is from about 2.5 Y 9/4 to about 2.5 Y 9/6 in the *Munsell Book of Color* when viewed in natural light.

54-55 (Cancelled)

56. (Previously presented) The seed of claim 51, wherein said seed germinates in an environment free of light.

57. (Previously presented) The seed of claim 51, wherein said seed is from the middle of a pod and is cuboid in shape.

58. (Previously presented) The seed of claim 51, wherein the dry seed weight is about 43 grams per 100 seeds (adjusted to 12 percent moisture).

59. (Previously presented) Seed from a field bean variety of *Phaseolus vulgaris* having a seed coat that is yellow in color, wherein the yellow color plotted as a distribution in a population of the seed of sufficient number for purposes of ATCC deposit has a peak occurrence ranging from about 7.5 Y 8.5/4 to about 7.5 Y 8.5/6 in the *Munsell Book of Color* when viewed in natural light.

60. (Previously presented) The seed of claim 59 comprising a hilar ring.

61. (Previously presented) The seed of claim 60 wherein the color of the hilar ring is from about 2.5 Y 9/4 to about 2.5 Y 9/6 in the *Munsell Book of Color* when viewed in natural light.

62. (Previously presented) Seed from a field bean variety of *Phaseolus vulgaris* having germplasm for expressing a seed coat that is yellow in color as evidenced by a substantially uniform yellow color of the seed coat, wherein the substantially uniform yellow color plotted as a distribution in a population of the seed of sufficient number for purposes of ATCC deposit has a peak occurrence ranging from about 7.5 Y 8.5/4 to about 7.5 Y 8.5/6 in the *Munsell Book of Color* when viewed in natural light.

63. (Previously presented) The seed of claim 62 comprising a hilar ring.

64. (Previously presented) The seed of claim 62 wherein the color of the hilar ring is from about 2.5 Y 9/4 to about 2.5 Y 9/6 in the *Munsell Book of Color* when viewed in natural light.

65. (Withdrawn) A population of seeds from a field bean variety of *Phaseolus vulgaris* wherein at least 70% of the seeds in the population have a hilar ring with a color from about 2.5 Y 9/4 to about 2.5 Y 9/6 in the *Munsell Book of Color* when viewed in natural light.

66. (Withdrawn) A population of seeds from a field bean variety of *Phaseolus vulgaris* wherein at least 85% of the seeds in the population have a seed coat with a color from about 7.5 Y 8.5/4 to about 7.5 Y 8.5/6 in the *Munsell Book of Color* when viewed in natural light.

67. (Withdrawn) The seed population of claim 66, wherein at least 70% of the seeds in the population have a hilar ring with a color from about 2.5 Y 9/4 to about 2.5 Y 9/6 in the *Munsell Book of Color* when viewed in natural light.

68. (Withdrawn) The seed population of claim 66, wherein the seed population originates from a population of pods, wherein at least 40% of the pods in the pod population have a beak orientation that is selected from the group consisting of straight, curved upward and combinations thereof.

69. (Withdrawn) The seed population of claim 66, wherein the seed population originates from a population of plants having leaflets, wherein at least 46% of the leaflets of the plant population have an ovate shape.

70. (Withdrawn) The seed population of claim 66, wherein the seed population originates from a population of plants having leaflets, wherein at least 61% of the leaflets of the plant population have a color of about 5 GY 5/6 in the *Munsell Book of Color* when viewed in natural light.

71. (Withdrawn) A method of producing a progeny field bean plant comprising crossing a first parent field bean plant with a second parent field bean plant, wherein the second parent field bean plant is the field bean plant of claim 2, the

progeny field bean plant produces a seed having a seed coat color of about 7.5 Y 8.5/4 to about 7.5 Y 8.5/6 in the *Munsell Book Of Color* when viewed in natural light.

Evidence Appendix

1
http://www.mun/ca/biology/scarr/4241F_Quantitative_Genetics.html
July 18, 2005 printout
Record Location: Appendix A of October 14, 2005 RCE

2
Declaration of Gil Waibel
Record Location: IDS of November 15, 2002, cite #1

3
Polly Proctor Declaration
Record Location: Submitted with Response of June 2, 2004

4
Pod-Ners' Response to Defendants' Motion to Compel
Record Location: IDS of November 15, 2002, cite #100

5
Larry Proctor Declaration including 7.5Y page of Munsell Book
Record Location: Submitted with October 14, 2005 RCE

6
Bassett et al., ASHS Journals Online (2000)
Record Location: IDS of June 3, 2002, cite #42

7
Pallotini et al. (Crop Science 44: 968-977, 2004
Record Location: Office Action of April 14, 2005, cite #U

8
Laura Conley Declaration
Record Location: IDS of November 15, 2002, cite #17

9
Kaplan (Guitarrero Cave, p. 146, 1980)
Record Location: Cited in Request for Reexamination, entered by Examiner at Office Action of September 25, 2002

10
Hernandez-Xolocotzi (Seminar Series 2E, CIAT, p. 253-258, 1973)
Record Location: Cited in Request for Reexamination, entered by Examiner at Office Action of September 25, 2002

11
Voysest (Varieties of Beans in Latin America, CIAT, p. 47-50, 1983)
Record Location: Cited in Request for Reexamination, entered by Examiner at Office Action of September 25, 2002

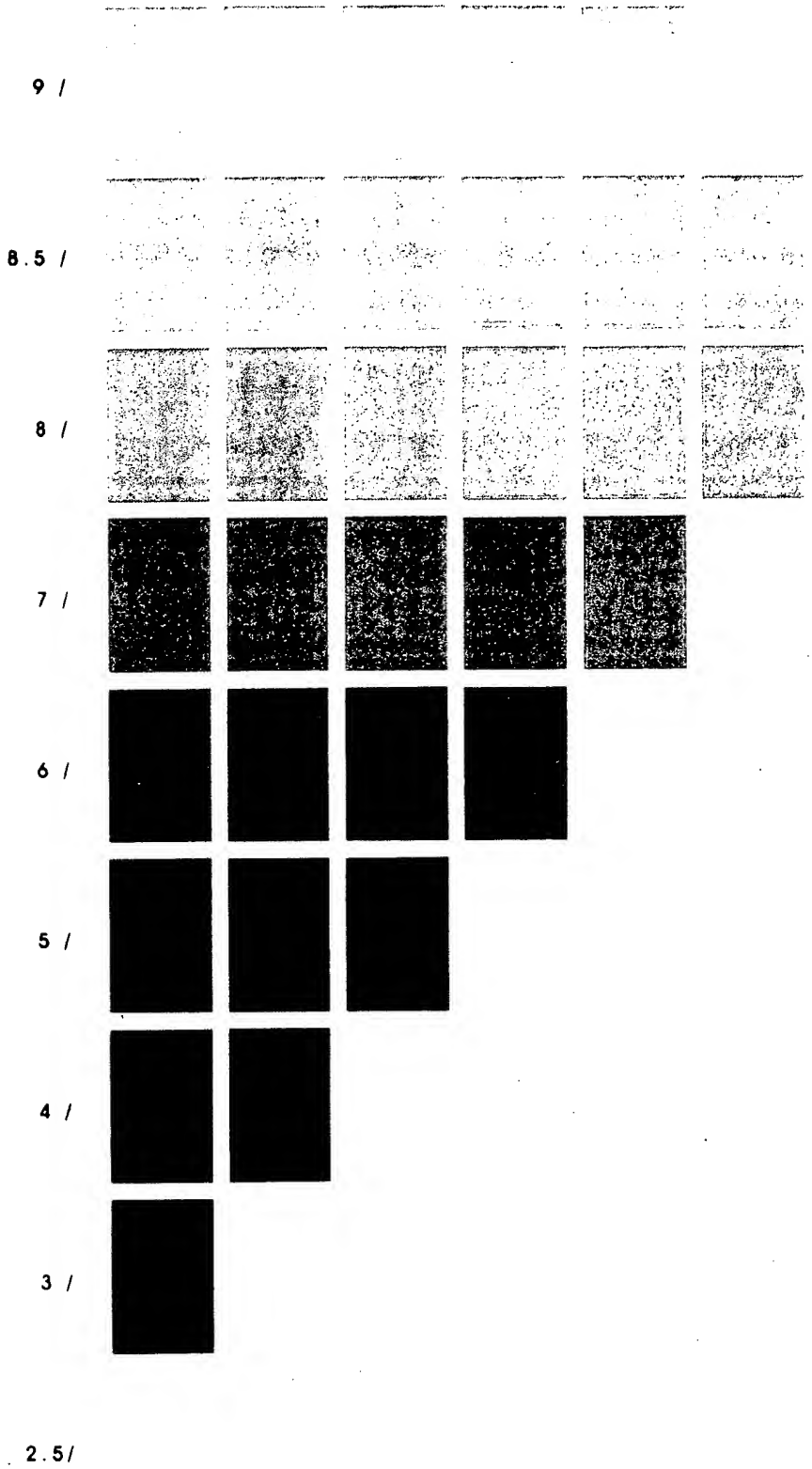
Related Proceedings Appendix

(BLANK)

The Munsell Book of Color

MATTE COLLECTION

Hue **7.5Y**



Quantitative Genetics

http://en.wikipedia.org/wiki/Quantitative_genetics

Sir Ronald Aylmer Fisher
http://www-gap.dcs.st-and.ac.uk/~history/Mathematicians/Fisher.html
http://instruct.uwo.ca/zoology/441a/hist3.html

Sewall Wright
http://instruct.uwo.ca/zoology/441a/hist4.html
http://www.amphilsoc.org/library/mole/w/wrights.htm

J.B.S. Haldane
http://instruct.uwo.ca/zoology/441a/hist5.html

Quantitative variation

Discontinuous traits: Traits that have only a few distinct phenotypes
Continuous traits: Traits that have continuous distributions of phenotypes

Quantitative vs Mendelian genetics:

Biometricians
- most variation in evolution didn't follow Mendelian rules
- continuous / blending variation that varies with environment
- Favors adaptation and survival of the fittest
- Mendelian traits are trivial and not interesting

Mendelians
- dismissed biometricians: genotype = phenotype?
- environmentally influenced = not inherited
- evidence supporting Mendelian genetics

Variation **is** continuous and can be inherited...
Continuous variation can be due to:
1. Numerous genes affecting expression (additive)
2. Environmental factors affecting expression (norm of reaction)
3. Both!

Multiple-factor hypothesis: many genes produce additive effect.
Polygenes: factors with small, equal effect
Polygenic traits: influenced by genetic variation at many loci
- analysis cannot be done by simple Mendelian genetics
- must compare phenotypic expression in close relatives (known to share a proportion of genes)

So what's environmental and what's genetic???

First a closer look **norm of reaction**...

Norm of Reaction

- relationship between environment and phenotype
- many phenotypes from one genotype under different environmental conditions
- phenotypes are constant in an environment (heritability measurements only apply to that environment)

To develop norm of reaction:
1. Develop a homozygous or stable heterozygous line (cloning, selfing)
2. Allow different lines to develop in different environments

Norm of reaction curves
- Plotting the phenotype of one genotype in each environment
- Determines the phenotypic distribution of the trait

Heritability of quantitative traits

Heritibility: the proportion of phenotypic variation due to genetic variation
Heritable - shared genotype
Familial - shared by a family (environmental)

But must account for environmental variation!!!!

Phenotypic variance (S_p^2) is due to genetic (S_g^2) and environmental (S_e^2) variance

$$S_p^2 = S_g^2 + S_e^2$$

These variances can also be broken down to include:
- Additive genetic variance (alleles contributing to the genotype)
- Dominance variance (heterozygotes not always an intermediate phenotype)
- Interaction variance (epistatic interactions)
- Environmental variance: general effects, reversible effects and maternal effects
- Covariance between an environment and a genotype
- Genetic-environment interaction

Heritability, H^2 is the portion of the overall phenotypic variance due to genetics

$$H^2 = S_g^2 / S_p^2$$
$$H^2 = S_g^2 / (S_g^2 + S_e^2)$$

Non-zero heritability means genetic differences effect trait expression
Perfect heritability (ie: a high H^2) does not mean that the environment does not play a part in the variation

Limits of H^2...
- Limited prediction of the effect of environmental modification (better with norm of reaction)
- Separation of variance into genetic and environmental components doesn't separate the genetic and environmental causes of variation [http://www.mun.ca/biology/scarr/fig27-14.htm]
- high heritability does not mean that a trait is unaffected by its environment.

A trait may have perfect heritability and still change due to environmental variation
Example: IQ scores in adopted children, and their parents

Children	Biological parents	Adoptive parents
110	90	118
112	92	114
114	94	110
116	96	120
118	98	112
120	100	116
Mean = 115	**Mean = 95**	**Mean = 115**

Different Types of Variance That Affect Heritability

Heritability in the broad sense has limits on its usefulness.

We can examine heritability in the narrow sense.

Additive genetic variance: due to average differences between carriers of alleles of a QTL.

Dominance variance: due to fact heterozygotes are not always exact intermediates between homozygotes.

More than one locus acting on a character->*epistatic interactions*

These interactions will appear as *Interaction variance* (s^2_i).

interaction variance is included in dominance variance (nonadditive variance).

Genetic variance is the sum of additive and dominance variance:

$$s^2_g = s^2_a + s^2_d$$

So phenotypic variance:

$$s^2_p = s^2_a + s^2_d + s^2_e$$

More narrow sense of heritability: ratio of additive variance to the phenotypic variance:

$$h^2 = s^2_a / s^2_p$$

Whats the difference?!?

Heritability*(Broad Sense)* $\mathbf{H^2}$: Phenotypic variance due to genetic variance.

Heritability*(narrow Sense)* $\mathbf{h^2}$: Phenotypic variance due to **additive variance alone.**

Estimating Components of Genetic Variance

Hard to estimate all the components of genetic variance.

Easy way to estimate h^2.

Plot phenotype of offspring vs midparent value.

Midparent Value: Average phenotype of both parents.

- Regression line passes through mean of parent and offspring.
- Slope is positive.
- Slope is less than unity.
- Slope reprsesents heritability!!
- Heritability isn't perfect, so neither is slope!

Selection differential: Difference between parents and mean of entire population in the same generation.

Selection response: Difference between offspring and mean of entire population in the same generation.

Selection response = h^2 x Selection differential.

If we know other two can rearrange to solve for h^2!

Done with selective breeding.

h^2 will not be same for different populations in different environments.

Environment plays a role that can't be ignored!

h^2 and breeding

h^2 used widely in commercial breeding.

Good to make strong lines by selecting for or against traits.

Look for group with a lot of genetic variance for that trait.

Why?

- Group with a lot of genetic variance has high h^2
- Higher h^2 = higher parent-offspring correlation!
- Larger fraction of offspring will have wanted trait from parents!

Sometimes forced to work with low h^2.

When h^2 & H^2 both low:

- Lots of environmental variance!
- **Family selection** used!
- Pairs produce trial offsrping, rather than just best individuals.
- Parents selected from progeny that do best.
- Cancels out some of environmental variation.

When h^2 but H^2 high:

- Little environmental variance!
- More dominance variance than additive variance.
- Make use of nonadditive variance.
- **Hybrid-inbred method.**
- Lines made by self crossing.
- These lines are then crossed.
- Choose best hybrid from these crosses.
- Selects for both additive and dominance variance.

Experience with corn makes effectiveness questionable!

Locating Genes

Its not always possible to locate all the genes that influence a character!

- Only some subsets within a population will be variable.
- Only some variation is actually noticable (i.e Blood group genes).

- Interference from environment can cloud phenotypes.

Genetic analysis only detects a gene if there's variation at that loci.

Molecular analysis can examines DNA and the information it translates!

- We can than look at changes in stretches of DNA, whether phenotype varies or not
- Great for comparing different species!

candidate gene: known loci that *may* be responsible for *some* of the phenotypic variance.

Marker Gene Segregation

quantitative trait loci (QTL): Loci whose alleic differences cause variance in a character.

These quatitative trait loci cannot always be identified.

It is possible though to try and track down a region of a chromosome where these QTL's lie.

- cross two lines that differ markedly in QTL's as well as two well known "marker" loci
- If the marker gene is linked to the QTL we can use it to identify the QTL in the next generation.
- Marker genes can be used to "tag" regions of DNA where QTL's lie!

Linkage Analysis

Marker Gene segregation requires that marker genes are linked to the QTL.

Must be able to make parental lines differing in marker alleles.

Types of molecular polymorphisms used in linkage analysis:

- Restriction fragment length polymorphisms (RFLP's).
- Tandem repeats.
- Single-nucleotide polymorphisms (SNPs).

Such polymorphisms are very abundant.

Makes it likely that two lines will have some differences in known molecular marker loci.

Lines differing in quantitative traits have differences in polymorphisms as well!

How does Linkage analysis work exactly??

1. Make 2 lines differing in both marker loci and the quantitative trait in loci.

2. Cross the two lines.
3. Cross F1 to itself or backcrossed with parental line.
4. Measure offspring for quantitative phenotype.
5. Characterize genotype and marker loci.

Marker loci & QTL unlinked: All genotypes of the marker show the same average phenotype for the quantitative trait.

Marker loci & QTL are linked: Each marker genotype has different average phenotype.

Confused?

Think of it this way:

- Marker gene and QTL are unlinked: Independent assortment.
- Marker gene genotype has no bearing on the quantitative phenotype.
- Any marker genotype has the same spread of quantitative phenotypes with same average value.

But...

- If the two are linked then independent assortment doesnt occur!
- Certain alleles of the QTL will separate with certain genotypes of the marker.
- Average phenotype for the A allele is different than that of the a allele!
- Difference depends on strength of the QTL on the phenotype & tightness of the linkage.

If linked we now have a good idea what region the QTL lies in!!

Finding the gene(s) responsible is made much easier, but still a daunting task!!

The gene *may be one of many* influencing the trait, other genes may lie in different regions!

Works great in organisms like *Drosophila*, but human pedigrees are too small to work with!

LOD Scores

A **LOD score** is a type of statistic. that makes use of a probability ratio.

LOD scores are used to determine linkage distance.

The ratio is the probability of a birth sequence with a linkage of a certain distance divided by the probability of a birth sequence where the genes are unlinked.

The log of this ratio is then taken as a "LOD Score".

Linkage distances with the highest LOD scores are the best estimates of real linkage distances.

Working with logs a LOD score of 2.0 means that it is 100 times more likely.

An example of a LOD Score.

Multipoint Mapping

Multipoint mapping is an extension of linkage analysis.

Try to map loci in question against several markers at once!

Lets us know which side of marker loci is on!

LOD score consists of *combined* probabilities!

Makes it hard to compute, use computers!

Using only a few heterozygous loci can take monthes to analyze!!!

Othe tools used in quantitative genetics

Statistics

Statistical analysis is vital in quantitative genetics.

No clear cut borders for quantitative phenotypes, deal with a range of phenotypes.

For this reason analysis involves measures of central tendency and dispersion

Central Tendancy

Mean: Average value of a group.

Dispersion

Variance: How much something differs from the mean value.

Pedigrees

For comparing phenotypes between offspring we often use pedigrees.

A pedigree is basically a family.

Can be very useful in tracking quantitative phenotypes through multiple generations.

The small size of human pedigrees severly limits the accuracy of linkage analysis.

Today...

www.henrystewart.com/journals/hg/Software%2520review.pdf
http://content.karger.com/produktedb/produkte.asp?typ=fulltext&file=hhe49194

Questions?Email us!

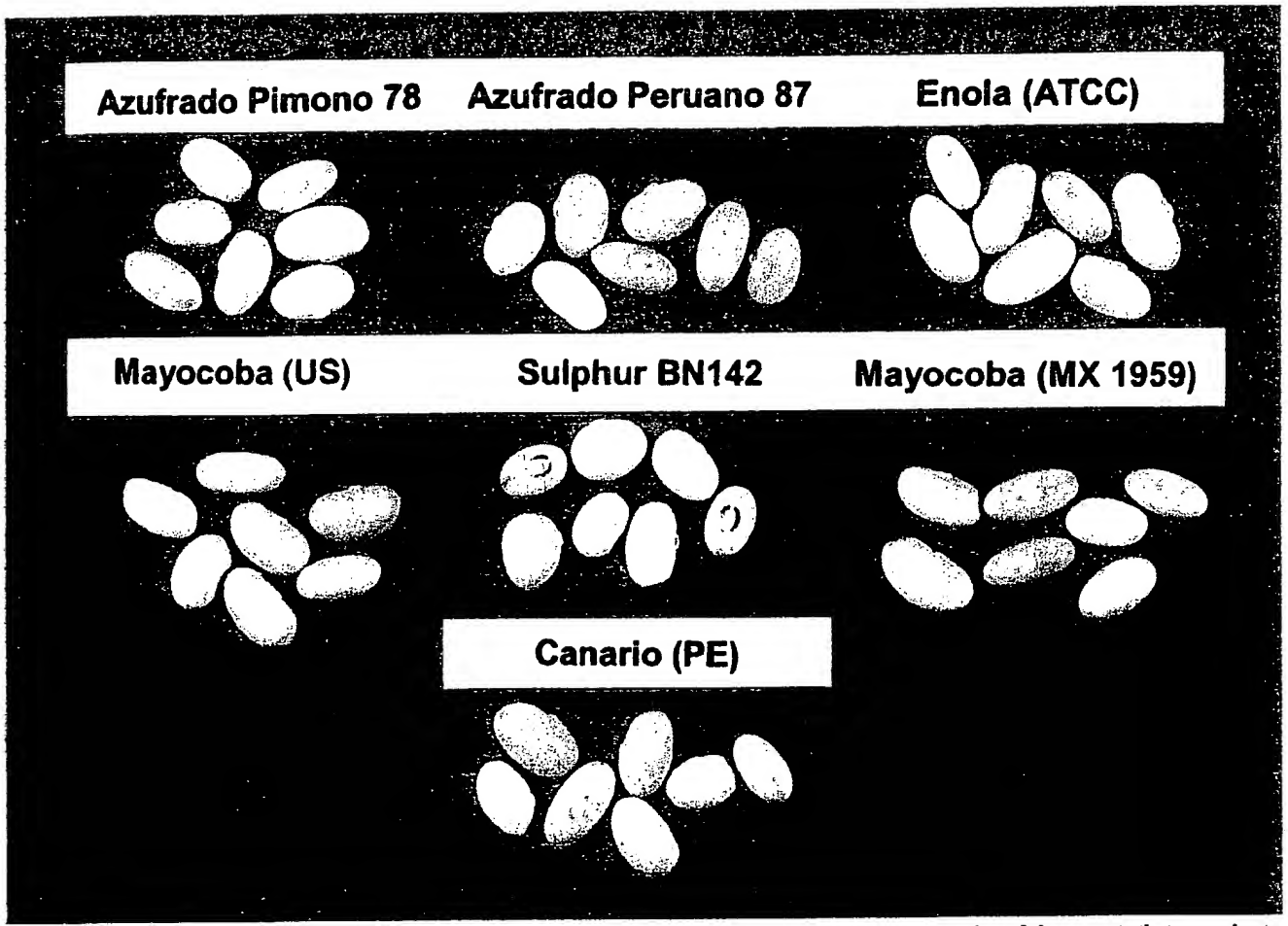


Fig. 1. Yellow-seed cultivars analyzed in this study. Mayocoba (MX 1959) and Canario (PE) are representative of the parents that gave rise to the Peruano type cultivar class. Examples of this class are the Mexican cultivars Azufrado Pimono 78 (the original Peruano-type cultivar released in 1978) and Azufrado Peruano 87 (released in 1987) (Voysest, 2000). Enola is a patented and PVP certified yellow-seeded cultivar (Proctor, 1999; http://www.ars-grin.gov/cgi-bin/npgs/html/acchtml.pl? 1536394; verified 27 January 2004). Sulphur BN142 is an ancient U.S. cultivar described as early as 1931 (Hedrick 1931).

Mexican cultivars belonging to the Peruano type commercial class, including Azufrado Pimono 78, the original cultivar in this class (Voysest, 2000), and Azufrado Peruano 87, a cultivar released in 1987 (Fig. 1); (ii) breeding lines in the Peruano category, such as SIN9 and SIN12; (iii) putative representatives of the Andean and Mesoamerican seed classes that gave rise to the Peruano class, Canario and G13094 (Mayocoba), respectively (Fig. 1); and (iv) the heirloom cultivar Sulphur BN142, described by Hedrick (1931) (Fig. 1).

In a second experiment, 15 individuals of each of three entries were analyzed. These entries included Enola (obtained from ATCC), Mayocoba (from a proprietary source), and Azufrado Peruano 87 (from INIFAP, Mexico). Results of the 15 individuals from this experiment were then combined with those of the respective individual analyzed in the first experiment, giving a total of 16 individuals for each entry. To allow a blind test in both experiments, individual accessions were given a consecutive number (Table 1) after receipt in the laboratory. This number was used throughout the experimentation and analysis of the results to allow a blind analysis of the results.

Amplified Fragment Length Polymorphisms

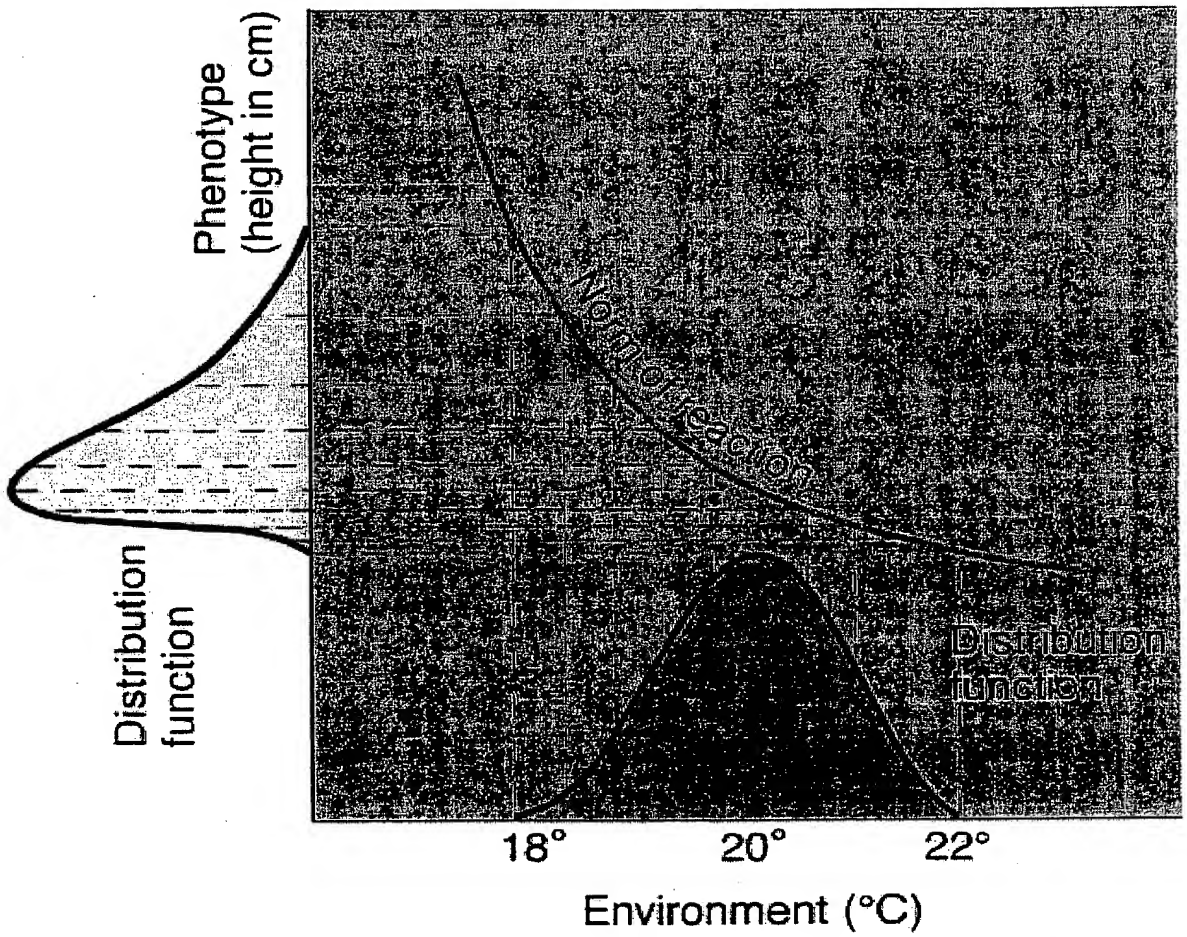
DNA was extracted from leaves harvested before flowering from greenhouse-grown leaves as described by Gepts and Clegg (1989), but without the addition of polyvinylpolypyrrolidone. Amplified fragment length polymorphisms were analyzed as described by Vos et al. (1995) and modified by Barcaccia et al. (1999). The primer combinations included five *Eco*RI-*Mse*I (with selective bases CAC/AAG, CAC/AGC, CCA/AGA, CCA/AGC, and CAA/AAG) and five *Pst*I-*Mse*I combinations (AG/CAC, AG/CAT, AG/CCA, AT/CAA, and AT/CAC).

To compare the efficiency of *Eco*I/*Mse*I and *Pst*I/*Mse*I primer combinations, an assay efficiency index (AI) was calculated (Table 2). The index relies on the effective number of alleles identified per locus, determined as $n_e = 1/\Sigma(p_i^2 + q_i^2)$, where p_i and q_i are the frequencies of marker alleles, present vs. absent, respectively at the ith locus. The index is then computed as $AI = (\Sigma n_e)/P$, where Σn_e is the total number of effective marker alleles detected over all polymorphic loci and P is the total number of assays performed (i.e., the number of primer combinations used) for their detection (Porceddu et al., 2002).

Data Analysis

Multivariate Analyses

In the first experiment, the principal coordinate analysis was implemented by, in succession, the SIMQUAL, DCENTER,


Phenotype
(height in cm)
Distribution
function
Distribution
function
18°
20°
22°
Environment (°C)

(2)

Declaration of Gil Waibel

Background of Gil Waibel:
B.S. degree in Agronomy from the University of Minnesota – 1975
Seed Analyst – USDA Seed Branch (Beltsville, MD and Minneapolis, MN) – 1975-78
Registered Seed Technologist (RST) – currently inactive
Owner / Seed Analyst – Teal Seed Lab – 1978-89
Co-Owner /Manager – Teal Farms (Dairy and Crop farm) – 1978-89
Seed Analyst and field inspector – Minn. Crop Improvement Association – 1989-91
Manager / Seed Analyst – Ferry Morse Seed Company – 1991-93
Seed Analyst – Asgrow Seed Company – 1993-96
President of the Idaho Seed Analyst Association 1996
Manager / Seed Analyst – Colorado Seed Lab – 1996-98
Member of AOSA Executive Board and Chair of the Publication committee – 1997-98
Manager – Colorado Seed Growers Association – 1998-02
Member of CSU Cultivar Release Committee – 1998-02
Taught at CSU:
SC380 "Plant and Seed Identification" 1997-01
Coached CSU Collegiate Crops Judging Team 1996-01
Member of CSU Seed Correspondence Group
Served as Chairman of the National Coaches Collegiate Crops Judging Committee
Owner / Manager Teal Dairy – 2002-present

While a field inspector for the Minnesota Crop Improvement Association, and Field inspecting for the Colorado Seed Growers Association, Waibel inspected many species including beans to check for trueness to variety. Each field inspected for seed certification, involved checking to see if the plants in the field were the variety stated to be planted in the field. As the field is walked, the field is also inspected for off-types.

While teaching the Plant and Seed Identification class, varietal differences were part of the material taught. Various varieties, or different plant types related to varietal differences were also covered in the class. The species where varietal differences were discussed were wheat, oats, rice and barley.

Preamble:
This project has been set up to determine if any or all of the plant and seed characteristics of yellow bean seed lots planted for Yellow River are clearly distinguishable from Enola. Have the PVP rights to the variety 'Enola" by Pod-Ners been infringed upon. The PVP application for Enola lists many plant and seed characteristics, which will be the bench-mark of this report. During the growing season of 2002, the plant and seed characteristics of Enola, and seed from Yellow River's 2001 Crop, and 2002 crop was observed and analyzed. In a perfect world, with every growing season being the same, seed quality being equal, and no micro-environments in the field, I would expect all characteristics of Enola to be the same as identified in the PVP application from 1996. Unfortunately, we do not have the luxury of equal growing seasons each year. Plants show slight differences in how they develop each year depending on the environment

they grow in. The Enola breeder seed lots tested (1996 and 2001 crop years) and Enola 2001 Certified seed lot were of seed certification genetic quality, yet the 1996 Enola (used as a bench-mark in this study) is getting old, and loosing vigor. The Yellow River seed used for crop year 2001 and 2002 was not certified seed, but commercial beans being used as seed. The Yellow River seed may be genetically inferior to the Breeder and Certified seed lots of Enola. It would be expected that some variability can exist between the Enola, and Yellow River's 2001 crop and 2002 crop lots in our studies due to the genetic differences that we are starting with.

Approach:
Originally we planned to have only one study (study 1) with various lots of seed from Pod-Ners, Yellow River, and Northern Feed and Bean. This study was going to be conducted at Hinesite Research in Delta, Colorado. It was set up as shown in attachment 1.

It was also decided to grow an identical plot in Greeley, Colorado so we could observe any differences that might occur due to the differing climate in Greeley from that of Delta, CO. Our plan was to have Kenneth Hines take notes on the growth and development of the plot in Delta, and have Gary Knight observe the same at the Greeley plots. Each man is an Agronomist with much experience in bean production. Neither man was told what the plots represented, except that all of the plots were yellow beans of one origin or another. We wanted to know if they saw any differences between the plots, but did not want them to have any bias in their observations. In some cases, very little seed was available, so to keep things equal, about 30 seeds were planted for each of three replicates at each site. The Greeley plot was planted May 31, and the Delta plot was planted June 9, 2002.

Over a week after we finished planting the Delta plot, we began to wonder if we had enough plants to make proper determinations about any/ or lack of distinguishing characteristics of the lots in question in this study. The plots were nice for observations, but we began to feel that the plant numbers were far less than we would need. We set up Study 3, which was comprised of samples of a 2001 Certified Seed lot of Enola, Yellow River 2001 commercial production "seed" lot sample, and Yellow River 2002 crop seed lot sample. Two replicates of 500 seeds each were planted at Hinesite Research in Delta. We wanted to plant three replicates, but space in the field did not allow for this. We did not replicate this study in Greeley. Study three was set up as shown in Attachment 2.

Gil Waibel traveled to Colorado to observe the plots in Greeley and Delta on July 14 to 16, 2002. He met with Kenneth Hines and Gary Knight and discussed with them what they should try to look for. Since they were to do their observation without knowledge of what each plot represented, they were instructed to make notes of any differences they might see. They were to watch for size differences, shape differences, color differences, plant architecture differences, rate of growth differences, and diseases symptoms that might occur, flowering dates, when pods form, maturity dates, and any other plant characteristic that might show itself.

It was decided that we needed to use the Munsell color charts on blooms, pods, leaflets (terminal), and mature pods. Our plan was to photograph (with the Munsell chart) as many color characteristics as we could. We decided after much practice photographing plots at the Greeley site, that we were not getting the detail we needed. We decided to photograph the plants flower by flower, leaflet by leaflet, and pod by pod. We began in photographing in Delta August 18-22, because it was a little farther along than the Greeley plots at that time. Due to the incredible amount of work to generate the date at Delta, it was decided the Waibel would go to Greeley to determine if data also needed to be drawn there as well. Waibel took a trifoliate of the two Enola lots, and placed the trifoliate into each plot at Delta. He saw no distinguishable difference between the Enola trifoliate and the trifoliates of the 2001 and 2002 crops from Yellow River in the Delta plot. The trifoliates used were placed into water, and placed into a zip-lock baggie for over night travel. Waibel drove to Greeley and, as was hoped, the trifoliates were in perfect condition. He place the Enola trifoliates into the 2001 and 2002 crop plots from Yellow River, and again saw no difference in color. It was Waibel's observation that on that day the plots in Greeley were very similar to the plots in Delta, and that all efforts should be concentrated in Delta to get as much data as possible.

We used a slide camera and a high-end (we thought) digital camera at Greeley July 30th. We hired a professional photographer, and he shot pictures for slides, and we shot the pictures with the digital camera. We wanted better results and hired another photographer (and color expert), who used a high quality digital camera. He shot the photos in Delta, and took the result back to his shop in Denver, and analyzed the color of the blooms, leaflets, and green pods. We did not dissect the blooms down to the standard, keel, and wings, because their color is the same. More photos and observations were made throughout September on the green phase in Delta. Mature observations and measurements were made in the last two weeks.

Results:

Plant Requirements - General

1. Market Classes

The PVP market class of Enola is number 12, Other (specify). The class specified is Yellow, of which Enola and Yellow River's 2001 crop and the 2002 crop lot are part of.

2. Maturity:

The PVP maturity of Enola is defined as late. Enola, and Yellow River's 2001 crop and 2002 crop lots matured in the "late" category as defined in the PVP application for Enola.

3. Days to maturity:

The PVP days to maturity of Enola is defined as 101 days.

With the exception of a couple of replicates, most replicates were at least 80% mature for study 1 by October 7, 2002 (120 days), and some replicates are still maturing. None of the replicates of study three were up to 80% mature by October 7. The maturity dates did vary from replicate to replicate, and until all of the maturity data is available, we cannot average the days to maturity for either study. The cool fall weather is slowing down the maturation process this year.

32. Suture color specific
The distribution of mature pod suture color for Enola, and Yellow River's 2001 crop and 2002 crop is similar. (refer to charts 14 and 30).

33. Cross section shape
The PVP application defines Enola as having a pear shaped cross section. In studies 1 and 3 the Enola and Yellow River's 2001 crop and 2002 crop lots were mostly pear shaped.

34. Curvature
The PVP application for mature pod curvature called Enola slightly curved. This years data showed a similar distribution toward curved for Enola and Yellow River's 2001 crop and 2002 crop with the exception of Yellow River's 2001 crop being equally split between slightly curved and curved. The pod curvature of results for Enola, and Yellow River's 2001 crop and 2002 crop are similar. (refer to charts 13 and 29).

35. Beak orientation
The PVP application for the mature pod beak orientation of Enola was defined as variable. Charts 10 and 26 show a very similar pattern between Enola and Yellow River's 2001 crop and 2002 crop lots.

36. Beak length
The PVP application defines the beak length of Enola as 1.2 cm. The pod beak length in study 1 and 3 is as follows:

	Study 1	Study 3	Average of studies 1 + 3
2001 crop	1.38 cm	1.18 cm	1.28 cm
2002 crop	1.19 cm	1.27 cm	1.23 cm
Enola	1.31 cm	1.21 cm	1.26 cm

37. Constrictions
The PVP application defines Enola as having slight mature pod constrictions. Charts 12 and 28 show a very similar pattern between Enola, and Yellow River's 2001 crop and 2002 crop lots – all showing slight constrictions.
38. Seeds per pod
The PVP application defines Enola as having 3.1 seeds per pod. Studies 1 and 3 showed the following seed per pod:

	Study 1	Study 3	Average of studies 1 + 3
2001 crop	2.46	4.2	3.34
2002 crop	3.74	3.8	3.78
Enola	2.74	3.2	2.98

Seed Morphology
39. Finish
The PVP application defines the seed finish of Enola as being semishiny. Enola, and Yellow River's 2001 crop and 2002 crop lots were all semishiny in seed finish.

40. Monochrome
The PVP application defines the seed of Enola as being monochrome in color. In studies 1 and 3, the seeds of Enola, and Yellow River's 2001 crop and 2002 crop lots were all monochrome in color.

41. Primary color general
The PVP application defines the primary color of Enola in general to be yellow. In studies 1 and 3, the seeds of Enola, and Yellow River's 2001 crop and 2002 crop lots were all yellow.

42. Primary color specific
The PVP application defines the seed coat color for Enola to be Munsell 5Y 8.5/4 to 7.5Y 8/8. In studies 1 and 3 (charts 31 and 15), most of the seed coat color for Enola, and Yellow River's 2001 crop and 2002 crop lots showed the PVP defined seed coat colors.

43. Color pattern
The PVP application defines the seed coat color pattern for Enola to be solid. In Studies 1 and 3, the seed coat pattern for Enola, and Yellow River's 2001 crop and 2002 crop lots were of a solid color pattern.

44. Hilar ring
The PVP application defines the seeds of Enola to have a hilar ring present. In studies 1 and 3, the seeds of Enola, and Yellow River's 2001 crop and 2002 crop lots all had hilar rings present.

45. Hilar ring color general
The hilar ring of Enola is specified as yellow in the PVP application. Our readings of hilar ring color are in number 46 because this report using the Munsell color scales.

46. Hilar ring color specific
The PVP application defined Enola's hilar ring color to be Munsell 2.5Y 9/4 to 2.5Y 9/6. Studies 1 and 3 (charts 32 and 16) showed very similar distributions of hilar color for Enola, and Yellow River's 2001 crop and 2002 crop lots in the color spectrum defined in the PVP application.

47. Shape
The PVP application defines the seed shape of Enola as cuboid. In studies 1 and 3, Enola, and Yellow River's 2001 crop and 2002 crop all have a seed shape of cuboid.

48. Weight 100 seeds
The PVP application defines the seed weight of Enola as 43 grams per 100 seeds. The data from studies 1 and 3 resulted in 100 seed weights as follows:

	Study 1	Study 2	Average of Studies 1 + 3
2001 crop	49 gm	54 gm	52 gm
2002 crop	55 gm	51 gm	53 gm
Enola	47 gm	58 gm	53 gm

Conclusion:

Most of the plant and seed characteristics between the Enola variety and Yellow River's 2001 crop and 2002 crop lots are close if not identical. Some of the slight characteristic differences and other issues need some discussion in this conclusion:

Average height at maturity:

Study 1 used seed from the 1996 breeder seed of Enola, and 2001 breeder seed of Enola. Breeder seed (genetically pure) does not have to meet the same certification seed quality standards as the Foundation, Registered and Certified seed classes meet. This seed may not have the same level of germination and/or vigor of the 2001 Certified lot of Enola planted in study 3. The results between Enola and the Yellow River 2001 crop and 2002 crop lots in study 3 are closer than the results in study 1.

Blossom color:

Blossom color was different than the PVP definition. There is a very light trace of pink in the blossoms. The color seems to deepen slightly as the day progresses. A quick look would cause the evaluator to say the blossom color is white. Even though this pink characteristic has not been identified in the PVP application, the Enola, and Yellow River 2001 crop and 2002 crop lots blossom color readings were very close.

Green leaflet color issue:

Lee Benson testified that he thought the color of the Enola field is different than the 2001 and 2002 Yellow River fields. As can be seen in chart 4 and chart 20, the leaf color readings for all three lots are very close. We found Benson's remark about the color to be compelling, so we took leaflet color readings in his field. The color distribution in his Enola and 2002 crop field is practically identical in chart 49. By the definition of the color of the green leaflets in the Enola PVP application, the color is the same.

Green pod constrictions:

Due to the rapid growth and development of bean pods in the green phase of the bean plant growth, the measure of constrictions is more meaningful in the mature stage. We did get similar distribution of green pod constrictions between the Enola, and Yellow River 2001 crop and 2002 crop lots.

Seeds per pod:

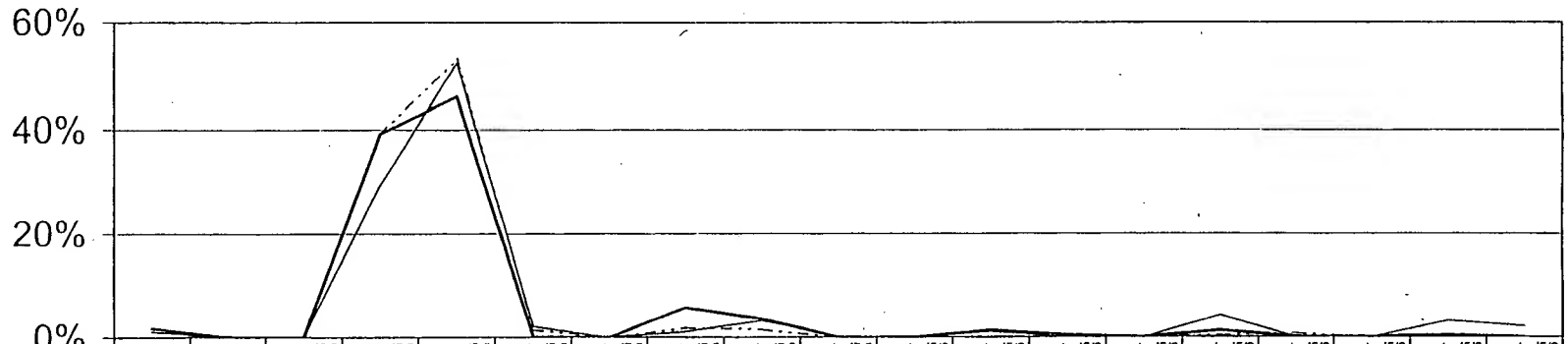
For some reason the seeds per pod for Enola is different than tha[illegible]r Yellow River's 2001 crop and 2002 crop lots. The Yellow river 2001 crop lot had the lowest count of seeds per pod in study one, and the highest number of seeds per pod in study three. Study one has the two breeder seed lots of Enola, one being the 1996 lot, and vigor seemed to be low in these two lots. Study three shows that there is little difference between Enola and Yellow River's 2001 crop and 2002 crop lots for seeds per pod.

It is my opinion after analyzing all of the data, that the Yellow River 2001 crop and the 2002 crop lots are not clearly distinguishable from that of the Enola variety.

Signature Gilbert P. Waibel Date 10/7/02

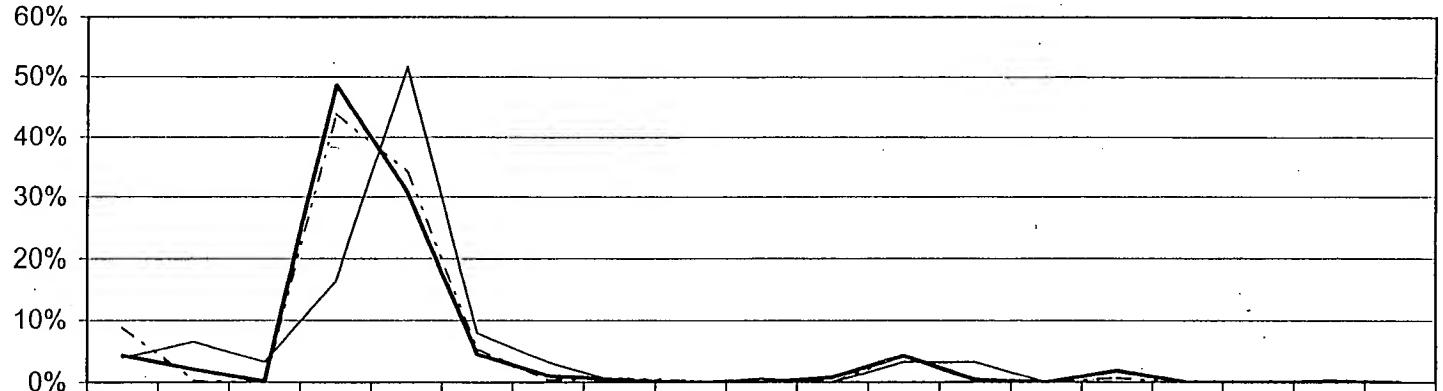
Gilbert P. Waibel

Chart 15 / Study 3 / Seed Coat Color



	scoatcol7.5 /9/4	scoatcol7.5 /9/6	scoatcol7.5 /9/8	scoatcol7.5 /8.5/4	scoatcol7.5 /8.5/6	scoatcol7.5 /8.5/8	scoatcol7.5 /8.5/10	scoatcol7.5 /8/4	scoatcol7.5 /8/6	scoatcol7.5 /8/8	scoatcol5/9 /2	scoatcol5/9 /4	scoatcol5/9 /6	scoatcol5/8 .5/2	scoatcol5/8 .5/4	scoatcol5/8 .5/6	scoatcol5/8 /2	scoatcol5/8 /4	scoatcol5/8 /6
2001 Crop	1%	0%	0%	29%	53%	2%	0%	1%	3%	0%	0%	1%	0%	.0%	4%	0%	0%	3%	2%
2002 Crop	0%	0%	0%	39%	53%	1%	0%	2%	1%	0%	0%	1%	0%	0%	0%	1%	0%	0%	0%
Enola	2%	0%	0%	39%	46%	0%	0%	6%	4%	0%	0%	1%	0%	0%	1%	0%	0%	0%	0%

Chart 31 / Study 1 / Seed Coat Color



	scoatcol7.5/9/4	scoatcol7.5/9/6	scoatcol7.5/9/8	scoatcol7.5/8.5/4	scoatcol7.5/8.5/6	scoatcol7.5/8.5/8	scoatcol7.5/8/4	scoatcol7.5/8/6	scoatcol7.5/8/8	scoatcol7.5/8.5/10	scoatcol5/9/2	scoatcol5/9/4	scoatcol5/9/6	scoatcol5/8.5/2	scoatcol5/8.5/4	scoatcol5/8.5/6	scoatcol5/8/2	scoatcol5/8/4	scoatcol5/8/6
——— 2001 Crop	4%	7%	3%	16%	52%	8%	3%	0%	0%	1%	0%	3%	3%	0%	0%	0%	0%	0%	0%
– - - – 2002 Crop	9%	0%	0%	44%	34%	5%	0%	1%	0%	0%	0%	4%	0%	0%	1%	0%	0%	0%	0%
——— Enola	4%	2%	0%	49%	31%	5%	1%	0%	0%	0%	1%	4%	0%	0%	2%	0%	0%	0%	0%

(3)

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

Reissue Application No. 09/773,303 | Group Art Unit: 1638
Filed: 31 January 2001 | Examiner: Nelson, Amy J.
Reexamination of U.S. Patent No. 5,894,079
For: FIELD BEAN CULTIVAR NAMED ENOLA
Inventor: Proctor, Larry M. | Date: 28 May, 2004

DECLARATION OF POLLY A. PROCTOR

1. My name is Polly A. Proctor. My address is P.O. Box 138, 1281 Pinion, Delta, Colorado 81416. I am related to the inventor of the cultivar involved in these proceedings, Larry Proctor, through marriage.

2. The statements that follow are based on my personal knowledge or, where stated, on my information and belief.

3. Larry and I have made best efforts to obtain beans cited by the Office from the repositories or other sources for the purpose of these proceedings.

4. I have received shipments of beans in fulfillment of the requests that Larry and I have made. To the extent that the requests have been unfulfilled, Larry and I regard these requests as being unfulfillable as, for example, where the repository cannot or will not release the beans.

5. I have personally tested each bean provided in these shipments to score the beans according to Munsel Color, and attach the results as Exhibit A to this Declaration. The testing was performed in a uniform way under conditions of

natural light and under witness by my colleague Kenneth Hines, whose address is 14782 D-Road, Delta, Colorado 81416.

I have closely reviewed and examined the test scores and compared them with the claims in U.S. Patent No. 5,894,079.

After careful examination, I conclude that none of the beans included in the documents provided by CIAT possesses the same Munsel Color as the beans claimed in U.S. Patent No. 5,894,079.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated this 28th day of May 2004.

Polly A. Proctor

Preliminary Color Comparison

2003 USPTO PI Accessions.xls
6/2/2004
5:36 PM

Exhibit A

PI 282060	Manteca	(Chile)	32 seeds						Munsell #	Qty
1	5Y 8.5/6	9	5Y 8/4	17	2.5Y 8/4	25	2.5Y 8.5/6		2.5Y 8/4	2
2	5Y 8/6	10	5Y 8/6	18	5Y 8.5/6	26	5Y 8.5/8		2.5Y 8/6	1
3	5Y 8/6	11	5Y 8/6	19	5Y 9/4	27	5Y 8/6		2.5Y 8.5/6	1
4	5Y 8/6	12	5Y 8/6	20	5Y 9/6	28	2.5Y 8/6		5Y 8/4	2
5	5Y 8/4	13	5Y 8/6	21	5Y 9/6	29	5Y 8/8		5Y 8/6	14
6	5Y 8/6	14	5Y 8/6	22	5Y 9/6	30	5Y 8.5/4		5Y 8/8	1
7	5Y 8/6	15	5Y 8/6	23	5Y 8.5/6	31	5Y 8.5/4		5Y 8.5/4	2
8	5Y 8/6	16	2.5Y 8/4	24	5Y 9/4	32	5Y 8/6		5Y 8.5/6	3
									5Y 8.5/8	1
									5Y 9/4	2
									5Y 9/6	3
PI 312090	Mantequilla	(Mexico)	34 seeds							
1	10Y 9/10	10	10Y 9/8	19	10Y 9/8	28	10Y 9/6		Munsell #	Qty
2	10Y 9/8	11	10Y 9/8	20	10Y 9/8	29	5Y 8.5/4		5Y 8.5/2	3
3	10Y 9/8	12	10Y 9/8	21	10Y 9/8	30	5Y 8.5/4		5Y 8.5/4	2
4	10Y 9/8	13	10Y 9/8	22	10Y 9/8	31	5Y 9/2		5Y 9/2	1
5	10Y 9/8	14	10Y 9/8	23	10Y 9/8	32	5Y 8.5/2		10Y 9/4	1
6	10Y 9/8	15	10Y 9/8	24	10Y 9/6	33	5Y 8.5/2		10Y 9/6	4
7	10Y 9/8	16	10Y 9/8	25	10Y 9/6	34	5Y 8.5/2		10Y 9/8	22
8	10Y 9/8	17	10Y 9/8	26	10Y 9/6				10Y 9/10	1
9	10Y 9/8	18	10Y 9/8	27	10Y 9/4					
NOTE: 7.5P color of the corona is found blended throughout the seed coat (Purple)										
PI 208777	G 1345	(Nicaragua)	45 seeds						Munsell #	Qty
1	5YR 8/6	12	5YR 6/8	23	2.5YR 6/6	34	5YR 6/8		10R 4/8	1
2	5YR 6/8	13	5YR 6/8	24	2.5YR 6/6	35	5YR 6/8		10R 6/6	1
3	5YR 7/8	14	5YR 6/6	25	2.5YR 6/6	36	5YR 6/8		2.5YR 5/6	3
4	5YR 6/8	15	5YR 5/6	26	2.5YR 5/8	37	5YR 7/6		2.5YR 5/8	2
5	5YR 7/8	16	5YR 7/6	27	2.5YR 5/6	38	5YR 7/8		2.5YR 6/6	7
6	5YR 6/8	17	5YR 6/6	28	2.5YR 5/8	39	5YR 4/6		5YR 4/6	1
7	5YR 6/8	18	5YR 6/8	29	2.5YR 6/6	40	5YR 5/6		5YR 5/6	3
8	5YR 6/8	19	5YR 7/6	30	2.5YR 6/6	41	2.5YR 5/6		5YR 6/6	3
9	5YR 7/6	20	5YR 7/8	31	10R 4/8	42	2.5YR 5/6		5YR 7/6	4
10	5YR 7/8	21	7.5YR 6/8	32	10R 6/6	43	2.5YR 6/6		5YR 8/6	1
11	5YR 6/8	22	2.5YR 6/6	33	5YR 5/8	44	5YR 5/6		5YR 5/8	1
						45	5YR 6/6		5YR 6/8	12
									5YR 7/8	5
									7.5YR 6/8	1

Comment: This was read and faxed on 11/22/02 by Polly

USPTO CIAT Accessions
2003 Samples

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	G 2400	Seed Coat					Hilar Ring					Seed Shape			Seed Size		Notations
2	1	10Y 8/8	11	10Y 8/8		1	7.5YR 5/8	11	7.5YR 5/8		20	Fastigiate		15	Medium		The Purple, Pink
3	2	10Y 8/8	12	10Y 8/8		2	7.5YR 5/8	12	7.5YR 6/6			Truncate		5	Small		Colors Scattered
4	3	10Y 8.5/8	13	10Y 8/8		3	7.5YR 5/8	13	7.5YR 5/8								Throughout The
5	4	10Y 8.5/8	14	10Y 8/8		4	7.5YR 5/8	14	7.5YR 5/8								Seeds: 2.5R page
6	5	10Y 8/8	15	10Y 8/8		5	7.5YR 5/8	15	7.5YR 5/6								Through 10R page
7	6	10Y 8/8	16	10Y 8/8		6	7.5YR 5/8	16	7.5YR 5/8								
8	7	10Y 8/8	17	10Y 8.5/6		7	7.5YR 5/8	17	7.5YR 5/8								
9	8	10Y 8/8	18	10Y 8.5/8		8	7.5YR 5/8	18	7.5YR 5/8								
10	9	10Y 8/8	19	10Y 8.5/8		9	7.5YR 5/8	19	7.5YR 5/8								
11	10	10Y 8/8	20	10Y 8.5/4		10	7.5YR 4/6	20	7.5YR 5/8								
12																	
13																	
14																	
15	G 11891	Seed Coat					Hilar Ring					Seed Shape			Seed Size		Notations
16	1	5Y 7/8	11	5Y 8/6		1	7.5YR 6/8	11	7.5YR 7/8		16	Cuboid		20	Small		
17	2	5Y 7/6	12	5Y 8/4		2	7.5YR 6/10	12	7.5YR 7/8		3	Kidney					
18	3	5Y 8/8	13	5Y 8/4		3	7.5YR 7/8	13	7.5YR 5/8		1	Truncate					
19	4	5Y 7/6	14	5Y 8/4		4	7.5YR 7/10	14	7.5YR 6/10								
20	5	5Y 8/6	15	5Y 8/4		5	7.5YR 6/8	15	7.5YR 6/10								
21	6	5Y 8/6	16	5Y 8/6		6	7.5YR 6/10	16	7.5YR 6/8								
22	7	5Y 8/6	17	5Y 8/6		7	7.5YR 6/10	17	7.5YR 7/8								
23	8	5Y 7/6	18	5Y 8/6		8	7.5YR 6/10	18	7.5YR 5/8								
24	9	5Y 8/6	19	5Y 8.5/4		9	7.5YR 5/8	19	7.5YR 6/8								
25	10	5Y 8/6	20	2.5Y 8/4		10	7.5YR 7/8	20	7.5YR 5/8								
26																	
27																	
28																	
29	G 13094	Seed Coat					Hilar Ring					Seed Shape			Seed Size		Notations
30	1	10Y 8.5/10	11	10Y 8.5/6		1	10YR 6/6	11	10YR 8/6		20	Cuboid to		5	Medium		
31	2	10Y 8.5/8	12	7.5Y 8/10		2	10YR 7/6	12	10YR 7/4			Slightly Kidney		15	Small		
32	3	10Y 8/8	13	7.5Y 8/10		3	10YR 8/6	13	10YR 8/6								

USPTO CIAT Accessions
2003 Samples

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
33	4	10Y 9/8	14	7.5Y 8/8		4	10YR 7/6	14	10YR 9/4								
34	5	10Y 8.5/8	15	7.5Y 8/6		5	10YR 7/6	15	10YR 8/6								
35	6	10Y 8.5/8	16	7.5Y 8/8		6	10YR 8/6	16	10YR 7/8								
36	7	10Y 8.5/8	17	7.5Y 8/6		7	10YR 6/6	17	10YR 7/8								
37	8	10Y 8.5/6	18	7.5Y 8/6		8	10YR 7/6	18	10YR 7/6								
38	9	10Y 8.5/6	19	7.5Y 8/6		9	10YR 7/6	19	10YR 7/6								
39	10	10Y 9/6	20	5Y 8/6		10	10YR 5/8	20	10YR 7/6								
40																	
41																	
42																	
43	G 22215	Seed Coat					Hilar Ring					Seed Shape			Seed Size		Notations
44	1	5Y 8/4	11	7.5Y 8/8		1	2.5Y 7/6	11	10YR 7/4		6	Round		16	Small		Was Very Hard
45	2	5Y 8/4	12	7.5Y 8/8		2	2.5Y 8.5/6	12	10YR 8/4		8	Oval		4	Medium		To Read Seed
46	3	5Y 8/6	13	7.5Y 8/6		3	2.5Y 8.5/8	13	10YR 7/4		3	Cuboid					Coat Color - Did
47	4	5Y 8/6	14	7.5Y 8/8		4	10YR 7/6	14	10YR 7/6		3	Kidney					Not Really Match
48	5	7.5Y 8/8	15	7.5Y 8/6		5	10YR 7/6	15	10YR 6/4								Any Color - Done
49	6	7.5Y 8/6	16	7.5Y 8.5/8		6	10YR 7/4	16	10YR 7/10								The Best Possible
50	7	7.5Y 8/6	17	7.5Y 8/8		7	10YR 7/4	17	10YR 6/6								
51	8	7.5Y 8/6	18	7.5Y 8.5/8		8	10YR 7/6	18	10YR 7/6								
52	9	7.5Y 8/6	19	7.5Y 8/8		9	10YR 8/6	19	10YR 6/6								
53	10	7.5Y 8/8	20	7.5Y 8.5/8		10	10YR 7/4	20	10YR 8/10								
54																	
55																	
56																	
57	G 22227	Seed Coat					Hilar Ring					Seed Shape			Seed Size		Notations
58	1	5Y 8/6	11	5Y 8/6		1	2.5Y 8.5/6	11	2.5Y 8/6		20	Cuboid		20	Small		
59	2	5Y 8/6	12	5Y 7/8		2	2.5Y 7/6	12	2.5Y 8/6								
60	3	5Y 8/8	13	5Y 8/8		3	2.5Y 8/8	13	2.5Y 7/8								
61	4	5Y 8/8	14	5Y 8.5/4		4	2.5Y 8/8	14	2.5Y 8/8								
62	5	5Y 8.5/6	15	7.5Y 8/4		5	2.5Y 8/8	15	2.5Y 8/6								
63	6	5Y 8/6	16	10Y 8/8		6	2.5Y 8.5/6	16	2.5Y 8.5/4								
64	7	5Y 8/6	17	7.5Y 8/8		7	2.5Y 8.5/6	17	2.5Y 8/4								
65	8	5Y 8/6	18	7.5Y 8/6		8	2.5Y 9/4	18	2.5Y 8.5/8								

USPTO CIAT Accessions
2003 Samples

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
66	9	5Y 8/6	19	7.5Y 8/4		9	2.5Y 8.5/8	19	10YR 6/10								
67	10	5Y 8/6	20	7.5Y 8/6		10	2.5Y 8/6	20	2.5Y 9/6								
68																	
69																	
70																	
71	G 22230	Seed Coat					Hilar Ring					Seed Shape			Seed Size		Notations
72	1	10Y 8/8	11	7.5Y 8/10		1	2.5Y 8/8	11	2.5Y 8/6		20	Cuboid		20	Medium		
73	2	10Y 8/8	12	7.5Y 8/12		2	2.5Y 8/8	12	2.5Y 6/6								
74	3	10Y 8.5/8	13	7.5Y 8/10		3	2.5Y 8/6	13	2.5Y 7/6								
75	4	10Y 8.5/8	14	7.5Y 8.5/10		4	2.5Y 6/6	14	10YR 5/6								
76	5	10Y 8.5/10	15	7.5Y 8/8		5	2.5Y 8.5/6	15	10YR 7/8								
77	6	10Y 8.5/8	16	7.5Y 8/6		6	2.5Y 8.5/10	16	10YR 5/8								
78	7	10Y 8.5/6	17	7.5Y 8.5/8		7	2.5Y 8.5/6	17	10YR 8/8								
79	8	10Y 8/6	18	5Y 8/8		8	2.5Y 8.5/8	18	10YR 6/8								
80	9	10Y 8.5/6	19	5Y 8/12		9	2.5Y 8.5/10	19	10YR 6/6								
81	10	7.5Y 8/6	20	5Y 7/8		10	2.5Y 8/8	20	10YR 5/6								
82																	

7.6

(4)

COPY

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF COLORADO

Civil Action No. 01 WY 2310 AJ (BNB)

POD-NERS, LLC, a Colorado limited liability company,

Plaintiff,

v.

NORTHERN FEED & BEAN OF LUCERNE LTD. LIABILITY CO., a Colorado limited liability company;
YELLOW RIVER, LLC, a Colorado limited liability company;
HIGHLAND FEED & BEAN, INC., a Colorado corporation;
FLYING K FARMS, INC., a Colorado corporation;
WHITMAN FARMS, INC., a Colorado corporation;
MURATA FARMS, LLC, a Colorado limited liability company;
LELAND BENSON, an individual;
LYNN FAGERBERG, an individual;
BARNARD GEISICK, an individual;
JIM GIBBS, an individual;
STAN HEINZE, an individual;
LOUIE FABRIZIOUS, an individual;
DENNIS KLEIN, an individual;
ROGER TROUDT, an individual;
CHUCK WINTER, an individual; and
STEVE WINTER, an individual,

Defendants.

POD-NERS' RESPONSE TO DEFENDANTS' MOTION TO COMPEL

TABLE OF CONTENTS

TABLE OF AUTHORITIES

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF COLORADO

Civil Action No. 01 WY 2310 AJ (BNB)

POD-NERS, LLC, a Colorado limited liability company,	)
	)
Pod-Ners,	)
	)
v.	)
	)
NORTHERN FEED & BEAN OF LUCERNE LTD. LIABILITY CO., a Colorado limited liability company; *et al.*	)
	)
Defendants.	)

POD-NERS' RESPONSE TO DEFENDANTS' MOTION TO COMPEL

Plaintiff Pod-Ners, LLC ("Pod-Ners"), by and through Lathrop & Gage L.C., its attorneys of record, hereby responds to Defendants' Motion to Compel Pod-Ners to Respond to Requests for Documents and Provide Testimony Improperly Withheld based on the Attorney-Client Privilege and Work Product Doctrine (hereinafter the "Motion" or "Motion to Compel") as follows:

b. Mr. Proctor Did Not Misrepresent His Beliefs Regarding the Variety Most Similar to the Enola Variety.

Defendants allege that Mr. Proctor knew that Azufrado Peruano 87 was the variety most similar to the Enola bean but told the PVP Office that Azufrado Pimono 78 was the variety most similar to the Enola bean. (Motion to Compel, p. 9.)

Again, Defendants rely solely on the testimony of Dr. Pfeiffer to support this alleged misrepresentation. Dr. Pfeiffer, however, testified that he did not know whether Peruano 87 was the variety closest to Enola.

> Q: What about the Peruano 87? Was that the closest out of – closest to the Enolas out of all of the varieties regardless of geographic location?
>
> A: I can't unequivocally say yes or no to that question because I don't know specifically the origin of the seed.

(Dr. Pfeiffer Depo., p. 287.)

Dr. Pfeiffer also explained that Pimono 78 was disclosed to the PVP Office rather than Peruano 87 because he did not have any Peruano 87 with which to compare to the Enola variety.

> Q: Why is it that you were able to tell the PVP Office that Pimono 78 was the closest?
>
> A: Because we had – originally the Bud originated from Mayocoba, as far as I know. And in the Mexican publication they

had listed Pimono 78 as a Mayocoba, and that's the only other thing that we had that we could make any possible comparison to.

Dr. Pfeiffer also testified that he attempted to get some Azufrado Peruano 87 seed in 1997 to make a color comparison with Enola. However, the seed that he obtained was treated and resulted in distorted and unreliable results. (Dr. Pfeiffer Depo., p. 300-01.)

Dr. Pfeiffer's uncertainty on the parentage was based in part on his experience that seed from Mexico, even allegedly registered seed, may not conform to its labeling.

Q: So how do you know what it is?

A: Oftentimes you don't. Even if it says it's a variety, you are not even quite sure that's, quote, the variety because I don't think the Mexican government maintains their seed stock from – . . .

(Dr. Pfeiffer Depo., p. 148.)

Q: What was your concern about comparing it to the parents?

A: Essentially didn't know what the parentage was. The seed that was planted in Delta, Colorado, I still don't know what the parentage of that was specifically, and even if it, again, was a variety that originated in Mexico, my theory on seed out of Mexico is that what you see – what you get and what's actually in the bag may be two totally different things.

(Dr. Pfeiffer Depo., pp. 281-82.)

In addition to Dr. Pfeiffer's lack of knowledge and information about Azufrado Peruano 87, at the time the PVP Certificate was being prosecuted, neither Mr. Larry Proctor nor Polly Proctor had any knowledge of the specific traits or characteristics of Peruano 87. (L. Proctor Dec., ¶ 22; P. Proctor Dec., ¶ 8.)

Again, in light of these facts, Defendants have made no showing that Mr. Proctor omitted any material information from the PVP Office regarding Peruano 87. Further, Defendants have made no showing that the PVP Office relied on any such omission and that the PVP Certificate would not have issued but for the omission.

c. Mr. Proctor Did Not Misrepresent the Percentage of the Enola Beans.

Defendants allege that Mr. Proctor knew that the beans were "believed" to have been developed from the Azufrado Peruano 87 and that he failed to disclose that fact to the PVP Office. (Motion to Compel, p. 9.)

As set forth in subsection (a) above, no one knew the exact parentage of the Enola bean. Failing to disclose Dr. Pfeiffer's suspicion that the parents may have been Peruano 87 is not a fraud, especially since Dr. Pfeiffer's suspicions were misplaced and groundless.

The beans that he sent to Mr. Proctor in 1994 for planting and which he believes were Peruano 87, were in fact marked as Pimono 78. (L. Proctor Dec., ¶ 17 and Exhibit 1.) Further, those beans did not germinate and were plowed under. (L. Proctor Dec., ¶

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

In re Reissue Application of Proctor
Reissue Application No. 09/773,303 — Group Art Unit: 1638
Filed: 31 January 2001 — Examiner: McElwain, Elizabeth F.
For: U.S. Patent No. 5,894,079 — Confirmation No. 6243

In re Proctor Reexamination Proceeding
Control No. 90/005,892
Filed: December 20, 2000
For: U.S. Patent No. 5,894,079
Title: FIELD BEAN CULTIVAR NAMED ENOLA — Dated: October 14, 2005

DECLARATION OF LARRY M. PROCTOR

1. My name is Larry M. Proctor. I reside at 2611 State Hwy 348, Delta, Colorado 81416. I am the sole named inventor on United States Patent No. 5,894,079 and am the President of Pod-Ners L.L.C,, which owns that patent. I am the same Larry M. Proctor that signed Declarations in this matter on March 25, 2003 and June 2, 2004.

2. I was involved in the Munsell color test work that took place before my application was filed. I did not conduct the tests myself (I found that my associates were better at it than I was) but I watched often and was thoroughly familiar with the Munsell Book of Color and the test methods we were using.

3. We found that the seed coat color of most of my Enola beans fell on the 7.5Y page of the Munsell Book. In addition, most of the beans matched one of two

color sample squares on the 7.5Y page. These were the 8.5/4 and 8.5/6 squares, which were next to one another. However, the match was not always perfect because the two Munsell squares we identified were just two single colors and the beans were not always those exact colors but somewhere in between.

4. The same was true of the colors of other parts of the Enola bean and plant. The hilar ring of most beans matched one of two color sample squares on the 2.5Y page. These were the 9/4 and 9/6 squares, which were next to one another. However, the match was not always perfect because the two Munsell squares we identified were just two single colors and the beans were not always those exact colors but somewhere in between.

5. The word "about" in my patent claims was put there to deal with this issue and was based mainly on the statement in the patent specification (twice) that "Enola seed possesses a unique yellow color matching most closely to 7.5Y 8.5/4 to 7.5Y 8.5/6 in the Munsell Book of Color when viewed in natural light" (col.3, ll. 31-34; col. 1, l. 65 to col. 2, ll. 4 (includes hilar ring)). I discussed this with my patent attorney. I hoped that people would not be able to avoid my claims with beans that were not the two exact shade of the two squares we specified.

6. It was not my intention, nor is it now, to stretch "about" to mean that a bean matching some other color square would be "about" the color discussed in my claims. If other beans had, for example, a seed coat color of 7.5Y 8.5/8 or 7.5Y 8/6, they would not in my view be within my claimed range of "about 7.5Y 8.5/4 to about 7.5Y 8.5/6" (see claim 13 for example). If I had been able to claim the 8.5/2 or 8/6 squares, I would certainly have done it. But my Enola beans did not fall there for the most part.

7. The color squares adjacent to my two claimed squares are markedly different colors. An accurate photographic copy of the 7.5Y page is attached to this Declaration as Attachment 1.

8. I understand that the Patent Office is continuing to ask for information about the public use or sale in the United States of the bean seeds I brought back from Mexico in the early 90's. I can state, and have stated, without hesitation that I have given the Patent Office all the information I can find on this issue, including bean samples that were near impossible to get and stacks of information developed in a contentious litigation with an infringer of my Plant Variety Protection Certificate.

9. I should note that I did not start looking for beans just because and when the Patent Office asked for information. I have been looking for similar beans since I started my work with Enola in 1991, and that effort intensified when we began to get ready to file applications for a patent and a Plant Variety Protection Certificate. Our attorney told us that it was very important to tell the Patent Office about everything we knew about, and maybe to look for more if we thought it was out there.

9. I refer the Patent Office to my prior Declaration in this matter (June 2, 2004) and to what I said there under penalty of perjury: "To the best of my knowledge, the beans purchased in Mexico by me were not in public use or on sale in the United States prior to the filing of my patent application."

10. I make these statements under penalty of perjury.

October 14, 2005

Larry M. Proctor

Attachment 1

Classical and Molecular Genetic Studies of the Strong Greenish Yellow Seedcoat Color in 'Wagenaar' and 'Enola' Common Bean

Mark J. Bassett[1]

Horticultural Sciences Department, University of Florida, Gainesville, FL 32611

Rian Lee,[2] **Carla Otto,**[3] **and Phillip E. McClean**[1]

Department of Plant Sciences, North Dakota State University, Fargo, ND 58105-5051

ADDITIONAL INDEX WORDS . *Phaseolus vulgaris*, seedcoat genetics, RAPD markers, Mayocoba dry beans

ABSTRACT . Inheritance of the strong greenish-yellow (SGY) seedcoat color in 'Wagenaar' common bean (*Phaseolus vulgaris* L.) was investigated. Line 5-593 is a determinate, Florida dry bean breeding line (with small black seeds) used as the recurrent parent in the development of many genetic stocks, e.g., *g b v* BC_3 5-593. Through crosses with genetic tester stocks, the seedcoat genotype of 'Wagenaar' was confirmed to be *C J g b* v^{lae} *Rk*. Three randomly amplified polymorphic DNA markers ($OAP7_{850}$, $OAP3_{1400}$, and $OU14_{950}$) that cosegregated with the *G* seedcoat color locus were developed from the F_2 population derived from the cross *g b v* BC_2 5-593 x *G b v* BC_3 5-593. From the cross 'Wagenaar' x *g b v* BC_3 5-593, 80 F_2 plants were classified into 54 non-SGY and 16 SGY seedcoat color plants. When the $OAP7_{850}$ marker was applied to that population, linkage was not observed with the non-SGY and SGY phenotypes. Conversely, a molecular marker ($OAP12_{400}$, that was developed from the F_2 from the cross 'Wagenaar' x *g b v* BC_3 5-593) linked to the locus controlling the SGY phenotype segregated independently of the *G* locus. Therefore, SGY phenotype is not controlled by the *G* locus. An F_3 progeny test of 76 F_2 plants from the cross 'Wagenaar' x *g b v* BC_3 5-593 confirmed the hypothesis that a single recessive gene (for which we propose the symbol *gy*) controls the seedcoat color change from pale greenish yellow (PGY) to SGY. Through crosses with genetic tester stocks, the seedcoat genotype of 'Enola' was determined to be *C J g b* v^{lae} *Rk*. The test cross 'Enola' x 'Wagenaar' demonstrated that 'Enola' also carries the *gy* gene. The relationship of 'Enola' to the 'Mayocoba' market class of common bean and to

'Azufrado Peruano 87' is discussed.

Prakken (1970) summarized the genetics of seedcoat colors in common bean (*Phaseolus vulgaris*) other than red colors, and he reconciled the various systems of gene symbols used by various researchers. Subsequently, Prakken (1972) published his extensive work with red seedcoat colors and organized the entire body of seedcoat color genetics (Prakken, 1970, 1972) into two tables, one for the yellow-black series of colors and the other (a text table) for the red colors. One of the colors in the first table is pale greenish yellow (canary), which was also called schamois by Lamprecht (1932). The genotype of pale greenish yellow is *P C J g b v Rk*. The seedcoat color investigated in the present paper is a very much more intense color, which we will designate as strong greenish yellow (SGY).

The seedcoat genotype of 'Wagenaar' was studied by Prakken (1940) in the cross 'Wagenaar' x 'Citroen' and found to be *P C D J g b* v^{lae} (using the gene symbols of Prakken, 1970). In subsequent work, Prakken (1972) used 'Wagenaar' in two diallel crossing sets of four parents each, and he analyzed the 12 resulting F_2 progenies. This work confirmed the previous genotype for seedcoat color, which is described as "shiny pale greenish yellow" with purple corona. The purple corona trait is controlled by the v^{lae} gene (Bassett, 1995a).

Prakken (1940) described 'Wagenaar' as a two-toned seedcoat (canary and schamois), which he interpreted as being always typical of the seedcoat genotype *P C J g b v* (or v^{lae}). The senior author of this paper has never observed expression of the canary yellow seedcoat color in the genetic stock *g b v* BC_3 5-593, which he describes as (very) pale greenish yellow (PGY). In this paper the canary color of Prakken (1940) will be called SGY.

When 'Wagenaar' is grown in the greenhouse at Gainesville, Fla., or Fargo, N. Dak., the distribution of SGY on the seedcoats of 'Wagenaar' is often incomplete, i.e., part of the seedcoat has PGY and the remainder SGY. Our observations for two-toned color pattern are the same as those of Prakken (1940), but our genetic interpretation is different. We hypothesize that the genotype *C J g b v* gives schamois to (very) pale greenish yellow, but never the SGY (canary) that Prakken (1940) observed in 'Wagenaar.' Furthermore, we hypothesize that an independent gene (tentative symbol *Gy*) with variable expressivity intensifies PGY to SGY in 'Wagenaar.' The same SGY color of 'Wagenaar' occurs with variable expressivity in the 'Mayocoba' market class of dry (common) bean (Bassett, unpublished observation). 'Enola', a patented dry bean cultivar, also has the SGY color with variable expressivity as shown in the color photograph included in the patent (Proctor, 1999). Therefore, the objectives of this work were to 1) determine the inheritance of SGY seedcoat color, 2) reexamine the full seedcoat genotype of 'Wagenaar', 3) test alternative hypotheses that propose that SGY is produced either by a mutant allele at the *G* (yellow seed color) locus or by a gene independent of *G*, using a combination of classical and molecular genetic approaches, 4) determine the seedcoat genotype of 'Enola' and the 'Mayocoba' market class, and 5) discuss the claims of the 'Enola' patent in relation to the findings of this paper.

Materials and Methods

Eight genes control seedcoat color in common bean, and very complicated epistatic interactions occur among those genes

Received for publication 18 Apr. 01. Accepted for publication 5 Sept. 2001. This research was supported by the Florida Agricultural Experiment Station and a T-STAR grant, and approved for publication as journal series no. N-01969. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

[1]Professor.

[2]Technician.

[3]Undergraduate student.

(Prakken, 1972). For this paper, only a brief introduction to the genetics of seedcoat color is needed. The cultivars tested in this paper all carried the dominant (wild type) allele at the *P*, *C*, *J*, and *Rk* loci, and those dominant alleles do not alter the color. Similarly, the cultivars tested all carried the recessive *r* allele at the *R* locus for dominant red color, which is closely linked to *C*. The genes *G*, *B*, and *V* are color modifying genes: *G* (from *Gelbe*, a German word) for yellow with *G b v*, *B* for mineral brown with *G B v*, and *V* for violet to black (anthocyanin pigments) with *G B V*. With *g b v*, the seedcoat is nearly colorless, shamois to (very) pale greenish yellow or cream color. This paper presents evidence for a ninth seedcoat color gene expressing SGY in the *P* [*C r*] *J g b v Rk* genetic background.

Seeds of 'Wagenaar' were obtained from H. Dijkstra, Collection Manager, Centre for Genetic Resources, Wageningen, The Netherlands. Prakken (1940) described a difference in the color of 'Wagenaar' "between the hilum side and the opposite dorsal (and lateral) side of the seed" as being characteristic. The hilum (ventral) side was canary yellow, whereas the dorsal side was schamois. The two color zones were not sharply separated, and transitional colors occurred. Prakken (1940) goes on to comment, "The canary yellow is extremely variable in its extension, even in seeds on the same plant; sometimes it is nearly imperceptible or restricted to very small ventral spots in the region of the germ root and near the caruncula; in other cases nearly the whole seedcoat can show the color." This exact and detailed description fits perfectly the observed seedcoat color of 'Wagenaar' when grown at Gainesville, Fla., or Fargo, N. Dak.

Seeds of the 'Mayocoba' market class were obtained from the Los Angeles market by a source that cannot be disclosed due to contractual agreement. Seeds of 'Enola' were obtained from Mark Brick, Colorado State University, Fort Collins. Our dry bean breeding line 5-593 (Florida) has small seed size, with shiny black seedcoats of genotype *T P* [*C r*] *D J G B V Rk* (Bassett, 1994, 1996a; Bassett and Blom, 1991; Prakken, 1970). Genetic tester stocks were developed by using 5-593 as the recurrent parent in backcrossing programs (with F_2 selection in each cycle) to transfer recessive genes, singly and in combination, into this standard genetic background. The genetic tester stocks used as testcross parents with 'Wagenaar' and 'Enola' are listed and described in Table 1.

Development of the genetic tester stock *g b v* BC_3 5-593 began with a cross of 'Calima' ([C^{ma} *R*] *J g b v Rk*) with *G b v* BC_2 5-593 (Bassett, unpublished data). From this cross, a true breeding F_3 progeny was developed with PGY seedcoat color ([*C r*] *J g b v*).

This F_3 was crossed with *G b v* BC_3 5-593 to create *g b v* BC_1 5-593. In a similar manner, two additional backcrosses (with F_2 selection in each cycle) to *G b v* BC_3 5-593 were used to create *g b v* BC_3 5-593 in Spring 1997.

Over the past several years, the genetic tester stocks P_3, P_5, P_6, P_8, and P_9 (Table 1) were all crossed with 'Wagenaar', and the F_1 progeny of the test crosses were grown in the greenhouse at Gainesville, Fla., to produce the most complete expression of the seedcoat genes involved. Data were recorded for flower color and seedcoat color and pattern of the F_2 seed produced. During the greenhouse season of 1999-2000, 'Enola' was crossed with genetic tester stocks P_1, P_3, P_4, P_7, and P_9 (Table 1), and the F_1 progeny of the test crosses were grown in the greenhouse at Gainesville, Fla. The F_2 progeny of the 'Enola' test crosses were grown in the field in Spring 2000. Flower color and seedcoat color and pattern phenotypes of the parental, F_1, F_2, and F_3 seed were recorded. Plants of 'Mayocoba' were grown in the greenhouse along side 'Enola' plants in 1999. Plant type and seed color data were recorded and used to further compare 'Mayocoba' and 'Enola'.

Test crosses for allelism of the SGY seedcoat color were made between 'Wagenaar' x 'Mayocoba' and 'Enola' x 'Wagenaar', and the F_1 progeny were grown in the greenhouse at Gainesville, Fla. Data were recorded on seedcoat color. From the cross 'Enola' x 'Wagenaar', seeds were harvested from 10 F_2 plants (bulked seed) selected for full SGY seedcoat color development. The SGY seedcoat color was characterized using two methods: 1) the Munsell Book of Color (1966 edition, 2.5R-10G, Munsell Color Co., Inc., Baltimore, Md.) and 2) a chromameter (model CR-200; Minolta, Ramsey, N. J.). For the latter technique, a seed sample was placed on a black table top as background. Data were recorded for both methods of characterization of SGY color.

The cross 'Wagenaar' x *g b v* BC_3 5-593 was made and 80 F_2 plants were grown in the greenhouse at Fargo, N. Dak. All seeds from each F_2 plant were harvested. The plants were also classified for flower color and seedcoat pattern. A complete genetic model for the phenotypic data was developed, and the genetic segregation data were analyzed using the orthogonal contrasts of Mather (1957). Genetic linkage was calculated by the maximum likelihood method and tables of Allard (1956).

All seeds from each F_2 plant from the cross 'Wagenaar' x *g b v* BC_3 5-593 were planted in the field at Gainesville, Fla., in Spring 2000 to achieve an F_3 progeny analysis of the F_2. Due to variable expressivity of the SGY trait, the seed sampling procedure varied depending on the phenotype of the F_2 parent. Prog

Table 1. Seedcoat phenotypes and genotypes of 'Wagenaar' and 'Enola' and the genetic stocks used in testcrosses to determine the seedcoat genotype of 'Wagenaar' and 'Enola' common bean.

Parent Stock		Seedcoat color		
no.	name	Phenotype	Genotype	Reference
P_1	Wagenaar	Strong greenish yellow with purple corona	*P C D J g b* v^{lae}	Prakken, 1972

P_2	Enola	Strong greenish yellow	Unknown	
P_3	c^u BC_3 5-593	Cartridge buff	*P* c^u *D J G B V*	Bassett, 1996a
P_4	c^u *b v* rk^d BC_1 5-593	Dark red kidney	*P* c^u *D J G b v* rk^d	Bassett, unpublished
P_5	[*c R*] *b v* BC_3 5-593	Oxblood red	*P* [*c R*] *D J G b v*	Bassett, 1996b
P_6	*j* BC_3 5-593	Dull dark purple with margo pattern[z]	*P C D j G B V*	Bassett, 1996a
P_7	*dj* BC_3 5-593	Dull dark purple with white hilum ring and corona	*P C dj G B V*	Bassett, 1996a
P_8	*G b v* BC_3 5-593	Yellow brown	*P C D J G b v*	Bassett, 1995b
P_9	*g b v* BC_3 5-593	Pale greenish yellow	*P C D J g b v*	

[z]Margo pattern has colored hilum ring, white (or nearly white) corona, and greater loss of dark purple color on the dorsal side of the seed than on the ventral side.

Table 2. Results of testcrosses between 'Wagenaar' common bean and a series of genetic stocks with known seedcoat genotypes.

Testcross[z]	Phenotype of seedcoats of seeds on F_1 plants from the testcross
P_1 x P_3	Black/cartridge buff marbled
P_1 x P_5	Red/yellow brown (with red haze) mottle (subtle). At first glance the seed looks all red like the tester. A low contrast mottle pattern not typical for the *C*/[*c R*] interaction, i.e., this is a true *C*/*c* mottle.
P_1 x P_6	Shiny black seed (no margo pattern)
P_1 x P_8	Violet (yellow brown with reddish haze)/pale yellow brown mottle (very subtle); purple corona
P_1 x P_9	Pale violet/pale greenish yellow mottle; purple corona

[z]The names, phenotypes, and genotypes of the parental lines (P_i) are given in Table 1.

enies from PGY F_2 parents had only one pod sampled and a single seed selected for the composite for that plot. Progenies from SGY F_2 parents had all pods harvested, and the entire seed production of each plant was classified for seedcoat color by scoring all seeds individually. F_3 plants that produced any seeds with SGY color were classified as SGY. There were 76 F_3 progenies grown, including a total of 670 plants. The mean F_3 consisted of 8.8 plants, with the range of 1 to 27 plants.

Following the procedures of Brady et al. (1998), randomly amplified polymorphic DNA (RAPD) markers were developed for two seedcoat color genes: *G* and a putative new gene

controlling SGY color and tentatively given the symbol *Gy*. The *G* locus markers were developed from the F_2 from the cross *g b v* BC_2 5-593 x *G b v* BC_3 5-593 (P_8). The genetic stock *g b v* BC_2 5-593 is an earliler backcross version of P_9 (Table 1). A RAPD marker for the *Gy* locus was developed from the F_2 population of the 'Wagenaar' x *g b v* BC_3 5-593 cross described above.

Results and Discussion

CHARACTERIZATION OF SGY SEEDCOAT COLOR. The observed color descriptors of the color tiles in the Munsell Book of Color most closely resembling the bulked seed sample from selected F_2 plants from the cross 'Enola' x 'Wagenaar' ranged from 5Y 8/6 to 8/10 and 7/8 to 7/10, and7.5Y 8/6 to 8/8 and 7/10. Those values describe the stronger greenish yellow of our selected seed materials compared with the paler greenish yellow of the seed materials described in the 'Enola' patent (Proctor, 1999). Using the same seed sample with the Minolta Chroma Meter, the objective color values recorded were L = 52.87, C = 36.13, and H = 86.9.

A NEW LOCUS FOR SGY VS. A MUTANT *G* GENE. Before this research, two competing hypotheses existed regarding the SGY seedcoat color phenotype. One hypothesis suggested the novel seedcoat color was the result of another allele at the classic *G* gene. Alternatively, it was hypothesized that the SGY seedcoat color phenotype was the result of a new gene that modified the PGY seedcoat color phenotype. Our first approach to testing the alternative hypotheses was to develop molecular markers linked to *G* and determine if they were linked to or cosegregated with the SGY phenotype.

An F_2 population (derived from the cross *g b v* BC_2 5-593 x *G b v* BC_3 5-593) segregating at *G* was used to identify a RAPD linked to the gene. The population segregated for yellow-brown (*G_*) and PGY (*gg*) seedcoat color in the expected 3:1 phenotypic ratio ($\chi^2_{3:1} = 1.67$, $P = 0.19$) for this dominant-acting gene. Two bulks were created, each consisting of a pool of eight DNA samples from the yellow-brown or PGY seedcoat color classes. The DNA bulks were amplified by polymerase chain reaction (PCR) using a series of primers, and three primers were discovered that produced amplification patterns in which a fragment was present in the yellow-brown (*G_*) but not the PGY (*gg*) seedcoat color bulks. DNA from each member of the segregating population was amplified with the three primers, and the fragment segregation patterns were identical. Each individual in the population that contained the fragment was the *G_* genotype, and each individual lacking the fragment was the *gg* genotype. Recombination was not observed; and, therefore, the three marker fragments, $OAP7_{850}$, $OAP3_{1400}$, and $OU14_{950}$, appear to cosegregate with *G*. In addition, the three markers cosegregated with respect to each other.

The next experiment tested the linkage between one *G*-linked marker ($OAP7_{850}$) and the SGY seedcoat color phenotype. An F_2 population from the cross 'Wagenaar' x *g b v* BC_3 5-593 (P_1 x P_9 of Table 2) segregated 3:1 for the PGY and SGY phenotypes (Table 3). Each individual in this population was scored for the presence and absence of the $OAP7_{850}$ marker. This *G* marker and the two seedcoat color phenotypes segregated independently in this population. We, therefore, hypothesized that the genetic factor controlling the SGY seedcoat color was not *G*.

The next step was to develop a molecular marker linked to a gene for the SGY seedcoat color phenotype. A bulk segregant procedure, similar to that described above for the *G* marker development, was used to discover marker $OAP12_{1400}$ that is linked in coupling with the dominant PGY phenotype. As described below, F_2 individuals were genotypically classified using F_3 family analysis. Using this data, the linkage between *Gy* and

Table 3. Segregation for seedcoat color in F_2 and F_3 from the cross 'Wagenaar' x *g b v* BC_3 5-593 (pale greenish yellow tester).

F_2 segregation			F_3 segregation				
No. of plants[z]	Seedcoat color	Genetic hypothesis	No. of progenies[y]	PGY[x] *Gy/-*	SGY[w] *gy/gy*	χ^2 3:1	*P*
69	PGY[x]	*Gy/-*	34	All			
			28	251	69	2.017	0.16
14	SGY[w]	*gy/gy*	14		All		

[z]For the F_2 segregation data 69 and 14, the $\chi^2_{3:1} = 2.928$, $P = 0.09$.

[y]For the F_2 segregation data 34, 28, and 14, the $\chi^2_{1:2:1} = 15.79$, $P < 0.001$.

[x]PGY = pale greenish yellow seedcoat.

[w]SGY = strong greenish yellow seedcoat.

Table. 4. Segregation for seedcoat color and pattern in the F_2 from the cross 'Wagenaar' (*C. J g b* v^{lae} *gy*) x *C J g b v Gy* BC_3 5-593.

No. of		Seedcoat		
plants[z]	Phenotype[y]		Genotype	
20	Violet, purple corona	*Gy/-*	*C/C*	$v^{lae}/-$
27	Violet/PGY mottled, purple corona	*Gy/-*	*C/C*[r]	$v^{lae}/-$
13	Violet, PGY corona	*Gy/-*	*C/C*	*v/v*
4	Violet/PGY mottled, PGY corona	*Gy/-*	*C/C*[r]	*v/v*
7	SGY, purple corona	*gy/gy*	-/-	$v^{lae}/-$
9	SGY, PGY corona	*gy/gy*	-/-	*v/v*

[z]Combining classes without regard for the *C* locus, the data 47, 17, 7, 9 give $\chi^2_{9:3:3:1} = 7.822$, $P = 0.05$. The orthogonal contrasts are $\chi^2_{Gy} = 1.067$, $P = 0.30$, $\chi^2_V = 2.400$, $P = 0.12$, and $\chi^2_L = 4.356$, $P = 0.037$; coupling linkage between *Gy* and *V* is 35.51 ± 6.938 cM.

[y]PGY = pale greenish yellow, SGY = strong greenish yellow.

the $OAP12_{1400}$ marker was determined to be 7.5 cM. To further test the independence of *G* and the gene controlling the SGY phenotype, the *G* segregating population was scored with the $OAP12_{1400}$ marker. The marker and the *G* locus segregated independently. These

molecular genetic tests lead us to the conclusion that the SGY phenotype derived from 'Wagenaar' is controlled by a genetic factor other than the *G* locus. At this point, the inheritance of this trait was studied in more detail.

'WAGENAAR' TEST CROSSES. 'Wagenaar' was crossed to a series of genetic tester stocks with known genotypes. The F_1 phenotypes (F_2 seed) are described below (Table 2). The cross with P_3 produced a marbled seedcoat with black and cartridge buff. The genetic interpretation is that 'Wagenaar' carries *C* (See, Bassett, 2000, Table 3, class 5). The interpretation of the test cross with P_3 is limited to determining that 'Wagenaar' does not carry c^u or the classic *c* allele of Lamprecht (1932). The 'Wagenaar' *C* gene, nevertheless, carries a linked mottling function, as will be presented and discussed below. The cross with P_5 produced a surprising result (Table 2). The expected yellow brown color had a red haze, for which no hypothesis is pursued in this paper. The genetic interpretation is that 'Wagenaar' carries *r*. The test cross with P_6 gave a nonallelic interaction, indicating that 'Wagenaar' carries *J* (Table 2). The test crosses with P_8 and P_9 gave allelic interactions at *G* and *B*, but not for the *V* locus. The presence of purple corona color indicates that 'Wagenaar' carries v^{lae}. Thus, the genotype of 'Wagenaar' has been confirmed to be *C J g b* v^{lae}, which is in agreement of the findings of Prakken (1972). Only the finding of the red haze over yellow brown in the test cross with P_5 is a new result. Prakken (1972) also established that 'Wagenaar' carries the gene *Rk* at the red kidney locus, and our results are consistent with that genotype.

The F_1 from the cross 'Wagenaar' x *g b v* BC_3 5-593 (P_1 x P_9 of Table 2) produced the PGY (in light pattern color areas of the seedcoat) of P_9. This result supports the hypothesis that the SGY of P_1 is a recessive trait. The segregation for seedcoat color in the F_2 from the cross P_1 x P_9 fit a 3:1 ratio for PGY seedcoats to SGY seedcoats, respectively (Table 3). The data were consistent with the hypothesis that SGY is controlled by a single recessive gene.

F3 TEST OF *Gy* HYPOTHESIS AND VARIABLE EXPRESSIVITY. The hypothesis of a single recessive-acting gene for SGY was tested in F_3. The 14 F_2 parents with SGY seedcoat color were true breeding in F_3 progenies (Table 3). The 28 F_2 parents with PGY seedcoats that segregated for PGY and SGY seedcoats in F_3 did so in a 3:1 ratio, respectively (Table 3). The remaining 34 F_2 parents with PGY seedcoats were true breeding. The above three classes of F_3 progenies failed to fit the expected 1:2:1 ratio for true breeding SGY, segregating progenies, and true breeding PGY progenies (Table 3). The failure was due to an excess of true breeding PGY progenies. Low F_2 seed yield probably prevented the accurate F_3 characterization of some PGY F_2 individuals as heterozygotes. The hypothesis that the SGY trait is controlled by a single recessive-acting gene is supported by two results from the F_3 test: 1) the SGY class was true breeding and 2) a 3:1 segregation ratio for PGY and SGY, respectively, observed for the heterozygous F_3 families.

We propose the gene symbol *gy* for the SGY trait. Although current rules for gene symbol

nomenclature in common bean usually require three letters for the gene symbol, an exception was made to avoid making strings of gene symbols for seedcoat color genotypes any longer than necessary. The Genetics Committee of the Bean Improvement Cooperative has approved the gene symbol *gy* for SGY trait. We avoided using the gene symbol *sgy* for good reason. Although seeds with genotype *C J g b v* have shamois color, in this paper we retained the name pale greenish yellow (PGY) in deference to the summary table of Prakken (1972). In a future revision of this table the color name for genotype *C J g b v* should be changed to shamois only. Apparently, Lamprecht never reported observing a seed stock with *gy* (Bassett, personal review of all Lamprecht papers), and Prakken never did genetic analysis with a seed stock having *C J g b v Gy*. Besides 'Wagenaar', the only other stocks with *g b* v^{lae} used by Prakken (1972) had C^{m} or C^{st} (both with *R* expressed in the dark pattern areas).

The SGY character showed variable expressivity in the F_3

Table 5. Results of testcrosses between 'Enola' common bean and a series of genetic stocks with known genotypes.

	Phenotypes of flowers and seedcoats on F_1 plants from the testcross	
Testcross[z]	Flower[y]	Seedcoat
$P_2 \times P_7$	C.V.	Shiny black seed (no margo pattern)
$P_2 \times P_3$	C.V	Dark mineral brown/cartridge buff marbling
$P_2 \times P_4$	P.P.	Yellow brown/cartridge buff marbling; no purple corona
$P_2 \times P_9$	P.P.	Violet/pale greenish yellow marbling; no purple corona

[z]The names, phenotypes, and genotypes of the parental lines (P_i) are given in Table 1.

[y]C.V. = cobalt violet color expressed by V/v^{lae}, P.P. = pale pink expressed by v^{lae}/v.

progenies derived from SGY F_2 parents. There was great variation from seed to seed within plants, from plant to plant within plots, and between plots for the frequency of extensively SGY colored seedcoats (data not presented). Although the data were not sufficient to develop a genetic model for the inheritance of higher and more stable expression of the SGY trait, the data suggested that other genetic factors may control higher expression levels.

For the cross 'Wagenaar' x *g b v* BC_3 5-593, the F_1 progeny had mottled pale violet/PGY seedcoats (Table 2), and the F_2 progeny segregated for the same phenotype (Table 4). This mottled phenotype was not expressed well in the F_3 progeny grown in the field, and no data were recorded for mottling in that generation. Although 'Wagenaar' has the dominant *C* gene (Table 2) (Bassett, 2000; Prakken, 1972), the mottling function (property) of the 'Wagenaar' *C* is designated by the symbol *C'* (Table 4). Interestingly, the mottling effect from *C'* does not express with *gy/gy*, and the SGY trait does not express (giving PGY by default) in the corona region with *gy/gy v/v* (Table 4). Thus, with *gy/gy* there is no seedcoat expression for the distinction between the *C/C* and *C'/C'* genotypes (Table 4). Two possible interpretations are that *Gy* may be 1) linked to *C* or 2) be an allele at *C*. Using the BAT 93 x

Jalo restriction fragment length polymorphism (RFLP) mapping system (Nodari et al., 1993), the sequence tagged site (STS) marker developed from the RAPD marker $OAP12_{1400}$ was mapped to linkage group B8, showing two map units between the *C* and *Gy* loci (McClean, personal communication). Thus, the data support close linkage, but not allelism at *C*. Similar procedures with the same mapping system demonstrated that *V* is located in linkage group B6. Hence, the weak linkage between *Gy* and *V* of about 35 cM (Table 4) was found to be artifactual (McClean, personal communication).

'ENOLA' TEST CROSSES AND RELATIONSHIP TO 'MAYOCOBA'. The cross P_2 x P_3 gave F_2 seeds with dark mineral brown/cartidge buff marbling, which is interpreted as a *C* gene in 'Enola' (Bassett, 2000) although a black/cartridge buff marbling is expected (Table 5). The cross P_2 x P_7 gave F_2 seeds with black color without pattern (a nonallelic response to the *j* in the tester), which is interpreted as a *J* gene in 'Enola' (Table 5). The cross P_2 x P_4 gave F_2 seeds with yellow brown/cartridge buff marbling with no purple corona, which is an allelic response for *b* and a nonalleleic response for rk^d and *rk* (Table 5). Surprisingly, the cross P_2 x P_9 gave F_2 seeds with violet/pale greenish yellow marbling and no purple corona, which is an allelic response for *g*, *b*, and *v* (Table 5). No hypothesis for the violet color will be pursued in this paper. The pink flower color of 'Enola' indicates that 'Enola' carries v^{lae} (Prakken, 1970). The cross P_2 x P_1 gave F_2 seeds with SGY color, which is an allelic response indicating that 'Enola' also carries *gy*. Thus, the seedcoat genotype *C J g b* v^{lae} *Rk gy* for 'Enola' has been demonstrated.

'Enola' has pink flowers that are known to result from expression of the gene v^{lae}, but the seedcoat does not have the purple corona color produced pleiotropically by v^{lae} (Bassett, 1995a; Prakken, 1970). Both the corona and hilum ring of 'Enola' are either SGY or PGY, whereas the hilum ring color produced by *C J g b v* is brown and by *C J g b* v^{lae} is dark purple (Prakken, 1970). The test cross 'Enola' x 'Wagenaar' produced F_2 seeds with SGY corona and hilum ring (data not presented). Similarly, the testcrosses P_2 x P_4 and P_2 x P_9 failed to show the expected purple corona, but, on the other hand, the flower color phenotypes for the four test crosses with 'Enola' support the hypothesis that v^{lae} is present in 'Enola' (Table 5). Our hypothesis is that 'Enola' carries an unknown, dominant epistatic gene that suppresses the expected dark purple corona and brown hilum ring.

Both 'Enola' and 'Mayocoba' have the same SGY seedcoat color as 'Wagenaar,' and both cultivars express the same SGY color in the corona and hilum ring in the presence of gene v^{lae}. The test cross 'Wagenaar' x 'Mayocoba' produced F_2 seeds with SGY color, purple corona, and brown hilum ring (data not presented). Thus, although 'Enola' and 'Mayocoba' both carry the *gy* gene for SGY, our hypothesis is that 'Mayocoba' carries an unknown, recessive epistatic gene that suppresses the expected dark corona and brown hilum ring in 'Mayocoba'. A full investigation of the interaction of v^{lae} (and the *C* and *J* genes for hilum ring color) with both dominant and recessive epistatic suppressor genes is beyond the scope of this paper.

Comparison of the plant structure of 'Enola' and the 'Mayocoba' stock used in this paper showed that they were virtually indistinguishable (data not presented). These appearance similarities are consistent with the hypothesis that 'Enola' is a selection from one of the

pure-line commercial cultivars of the 'Mayocoba' market class grown in Mexico for export to the United States market. In the 1960's, or by early 1970 at the latest, the land race Canario (same as U.S. 'Mayocoba' class) was sent from Peru to Mexico (O. Voysest, personal communication). This was the first time that 'Mayocoba' beans were introduced into Mexico. The Mexicans crossed 'Canario Divex 8120' x 'Canario 107' and released the derivative cultivar 'Azufrado Pimono 78' in 1978. This began a new commercial class in Mexico, which they designate "Peruano" (Voysest, 2000). In the early 1980's the name of 'Azufrado Pimono 78' was changed to 'Mayocoba'. This very popular Mexican cultivar name was, thereafter, used to denote the market class in the United States. More than five other Peruano cultivars were developed in Mexico after 'Mayocoba'. In 1987, Mexican bean breeder Ingeniero Salinas and colleagues released 'Azufrado Peruano 87', a Peruano class bean cultivar (Kelly, 2000). After the 'Enola' patent (Proctor, 1999) was issued, a biotechnology laboratory in Texacoco, Mexico, determined by DNA analysis of 'Enola' (seeds obtained from the U.S. Patent Office) that 'Enola' was derived from 'Azufrado Peruano 87' (Kelly, 2000).

The patent for 'Enola' (Proctor, 1999) claims that "Enola seeds possess a unique yellow color...", but the results given above demonstrate that the well-known 'Wagenaar' bean cultivar, as well as all the Peruano market class cultivars of Mexico, have the same seedcoat color. When 'Enola' and 'Mayocoba' are grown together in the same greenhouse in Gainesville, the SGY seedcoat color of both cultivars is strongly expressed and covers the entire seedcoat of nearly all seeds when plants mature in December; but when the same cultivars mature together in March, the seedcoat color is distributed incompletely on the seedcoat and is weaker in expression. This seasonal variation is also typical of 'Wagenaar' when grown in greenhouse culture and is typical for a trait with varible expressivity. The 'Enola' patent (Proctor, 1999) also claims that the yellow color of the seed remains uniform and stable from season to season., but our results do not support that claim. The 'Enola' patent (Proctor, 1999) makes an exclusive property claim to all bean cultivars with the seedcoat color of 'Enola' (referred to as SGY in this paper) based on "invention" of that seedcoat color, but we assert that the program (described in the patent) of several successive cycles of self-pollination and selection from yellow bean materials purchased in Mexico did not create or invent the seedcoat color of 'Enola', i.e., the "invention" has no basis in fact.

Literature Cited

Allard, R.W. 1956. Formulas and tables to facilitate the calculation of recombinational values in heredity. Hilgardia 24:235-278.

Bassett, M.J. 1994. The genotype for seedcoat color of breeding line 5-593. Annu. Rpt. Bean Improvement Coop. 37:244-245.

Bassett, M.J. 1995a. The dark corona character in seedcoats of common bean cosegregates with the pink flower allele v^{lae}. J. Amer. Soc. Hort. Sci. 120:520-522.

Bassett, M.J. 1995b. A new recessive allele at the *C* locus for seedcoat color in common bean. J. Amer. Soc. Hort. Sci. 120:896-899.

Bassett, M.J. 1996a. The margo (*mar*) seedcoat color gene is a synonym for the Joker (*j*) locus in common bean. J. Amer. Soc. Hort. Sci. 121:1028-1031.

Bassett, M.J. 1996b. A complex *C* region genotype [*? R*] that with *G B* v^{lae} produces dark seal-brown seedcoat color in common bean. J. Amer. Soc. Hort. Sci. 121:594-598.

Bassett, M.J. 2000. A test cross protocol for determining the seedcoat genotype at the *C* locus in common bean. HortScience 35:286-289.

Bassett, M.J. and A. Blom. 1991. A new genotype for white seed coat discovered in 'Early Wax' snap bean. J. Amer. Soc. Hort. Sci. 116:131-136.

Brady, L., M.J. Bassett, and P.E. McClean. 1998. Molecular markers associated with *T* and *Z*, two genes controlling partly colored seed coat patterns in common bean. Crop Sci. 38:1073-1075.

Kelly, J.D. 2000. Enola yellow bean patent. Michigan Dry Bean Dig. 24(3):2-3.

Lamprecht, H. 1932. Beiträge zur Genetik von *Phaseolus vulgaris*. Zur Vererbung der Testafarbe. Hereditas 16:169-211.

Mather, K. 1957. The measurement of linkage in heredity. 2nd ed. Wiley, New York.

Nodari, R.O., S.M. Tsai, R.L. Gilbertson, and P. Gepts. 1993. Towards an integrated linkage map of common bean. II. Development of an RFLP-based linkage map. Theor. Appl. Genet. 85:513-520.

Prakken, R. 1940. Inheritance of colours in *Phaseolus vulgaris* L. I. Genetica 22:331-408.

Prakken, R. 1970. Inheritance of colour in *Phaseolus vulgaris* L. II. A critical review. Meded. Landbouwhogeschool Wageningen 70-23:1-38.

Prakken, R. 1972. Inheritance of colours in *Phaseolus vulgaris* L. III. On genes for red seedcoat colour and a general synthesis. Meded. Landbouwhogeschool Wageningen, 72-29.

Proctor, L.M. 1999. Field bean cultivar named Enola. U.S. Patent No. 5,894,079 (13 Apr. 1999). U.S. Patent and Trademark Office, Wash., D.C.

Voysest, O. 2000. Yellow beans in Latin America. Michigan Dry Bean Dig. 24(3):4-8.

American Society for Horticultural Science
113 South West Street, Suite 200, Alexandria, VA 22314-2851, USA
Phone +703.836.4606; Fax +703.836.2024
ashs@ashs.org

(7)

PLANT GENETIC RESOURCES

The Genetic Anatomy of a Patented Yellow Bean

L. Pallottini, E. Garcia, J. Kami, G. Barcaccia, and P. Gepts*

ABSTRACT

Since a 1980 Supreme Court decision, it is possible in the USA to obtain a utility patent for crop cultivars and other life forms. Furthermore, it is also possible to obtain Plant Variety Protection (PVP) for a cultivar. Among the awards of the United States Patent and Trademark Office and the USDA Plant PVP Office are a utility patent and a PVP certificate, respectively, associated with a yellow-seeded bean (*Phaseolus vulgaris* L.), specifically the cultivar Enola. These awards have been controversial because of, among several reasons, the perceived lack of novelty of the yellow seed color and the cultivar itself. To check the origin of Enola, we fingerprinted a representative sample of 56 domesticated common bean accessions, including a subsample of 24 cultivars with yellow seeds similar to those of Enola. Fingerprinting was accomplished with amplified fragment length polymorphisms (AFLP). Five *Eco*RI/*Mse*I and five *Pst*I/*Mse*I primer combinations were used, which revealed 133 fragments. The *Pst*I/*Mse*I primer combinations revealed a 3-fold larger number of polymorphic markers than the *Eco*RI/*Mse*I primer combinations. Most yellow-seeded beans, including Enola, were included in a tightly knit subgroup of the Andean gene pool. Enola was most closely related to the pre-existing Mexican cultivar Azufrado Peruano 87. A sample of 16 individuals of Enola displayed a single 133-AFLP-fragment fingerprint, which was identical to a fingerprint observed among yellow-seeded beans from Mexico, including Azufrado Peruano 87. Probability calculations of matching the specific Enola fingerprint showed that the most likely origin of Enola is by direct selection within pre-existing yellow-bean cultivars from Mexico, most probably 'Azufrado Peruano 87'.

In 1980, the U.S. Supreme Court instated (447 U.S. 303) the award of a utility patent for a genetically engineered *Pseudomonas* bacterium capable of breaking down crude oil (U.S. Supreme Court, 1980). An application for this patent had initially been rejected by the U.S. Patent and Trademark Office (PTO) on the ground that living things are not patentable subject matter according to the statute governing patents (Title 35 U.S.C. 101). The landmark Supreme Court decision initiated a new era in which patents for life forms, including DNA sequences, cell lines, transgenic animals and plants, and crop cultivars, could be obtained in the USA. Before this decision, the only life form for which a patent could be obtained were vegetatively propagated plants (so called plant patents). Utility patents are awarded for inventions that are novel, in that they must not have been made public for more than 1 yr. They should also be useful and nonobvious to someone skilled in the art (35 USC § 101, 102, 103) (U.S. House of Representatives, 2002).

In 1999, the U.S. PTO awarded patent no. 5,894,079 for the yellow-seeded cultivar Enola of common bean. The main claim of this patent is the yellow color of the seed coat of Enola. According to the patent description (Proctor, 1999), seeds of this cultivar had been obtained as part of a mixed bag of seeds of different colors purchased in Mexico in 1994. The yellow seeds were then planted in a field in Colorado for 3 yr (1994–1996) after which a patent for this yellow-seeded variety was filed on 15 Nov. 1996. Furthermore, the Plant Variety Protection (PVP) Office of the USDA issued PVP certificate no. 9700027 for cultivar Enola in 1999 (http://www.ars-grin.gov/cgi-bin/npgs/html/acchtml.pl?1536394; verified 8 January 2004). The award of these intellectual property rights has generated widespread attention in the media (New York Times: Pratt, 2001; National Public Radio: Tolan, 2001; Wall Street Journal: Friedland, 2000).

Yellow beans are among traditional bean cultivars grown principally in Mexico and Peru under several names such as Azufrado and Canario (Voysest, 2000). Originally, cultivars from these two countries represented two evolutionarily distinct groups of cultivars as they originated from two different domestications, one in Mexico and the other in the southern Andes (Gepts et al., 1986). More recently, Mexican bean breeders developed a new commercial class of yellow-seeded bean cultivars called Azufrado Peruano or Peruano by crossing yellow-seeded bean cultivars from Mexico with those of Peruvian origin (Voysest, 2000). Beans with yellow-colored seeds such as the Peruano types are grown and consumed mainly in the northwestern part of Mexico (Anonymous, 2000), but Mexican immigration has created a market for yellow-seeded beans in the USA.

We investigated here whether the Enola bean represents a distinct cultivar compared with the existing Peruano or other yellow-seeded cultivars from Mexico. We conducted DNA fingerprinting experiments to determine the relationships between Enola and other bean cultivars, including a sample of other yellow-seeded cultivars. We calculated probabilities of obtaining a match for the Enola fingerprint under different breeding scenarios. Furthermore, we compared the leaf color of Enola with that of selected yellow-seeded cultivars because

L. Pallottini and G. Barcaccia, Department of Agronomy and Crop Science, University of Padova, Agripolis, Via Romea 16, 35020 Legnaro, PD, Italy; E. Garcia, Department of Food Science and Technology, University of California, 1 Shields Avenue, Davis, CA 95616-8598, USA; J. Kami and P. Gepts, Department of Agronomy and Range Science, University of California, 1 Shields Avenue, Davis, CA 95616-8515, USA. Received 9 July 2003. *Corresponding author (plgepts@ucdavis.edu).

Published in Crop Sci. 44:968–977 (2004).

677 S. Segoe Rd., Madison, WI 53711 USA

Abbreviations: AFLP, amplified fragment length polymorphism; AP78, Azufrado Pimono 78; AP87, Azufrado Peruano 87; ATCC: American Type Culture Collection; INIFAP: Instituto Nacional de Investigaciones Forestales y Agropecuarias, Mexico; PRO: Proprietary source; PTO, Patent and Trademark Office; PVP, Plant Variety Protection; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; UC Davis: University of California, Davis.

the PVP application for Enola cited leaf color difference as a distinguishing mark between Enola and Azufrado Pimono 78, the original yellow-seeded cultivar of the Peruano type in Mexico. Our results show that the DNA fingerprint of Enola is identical to a fingerprint found in Mexican yellow-seeded beans of the Peruano group.

MATERIALS AND METHODS

Plant Materials

In a first experiment, a sample of 56 entries was established to investigate the relationships of cultivar Enola with other common bean cultivars (Table 1). One individual of each entry was analyzed. Some of the entries in this sample were received from more than location or more than once. Because they were analyzed separately, they were counted as a distinct entry. Enola seeds were obtained from the American Type Culture Collection, the official repository for patented cultivars, as well as from a private source. The six major races of common bean (Singh et al., 1991a) were represented by five to six accessions each, chosen on the basis of previous molecular markers analyses (Gepts, 1984, 1988; Singh et al., 1991b). In addition, special attention was devoted to assembling a sample of cultivars, whose seeds show a yellow color similar to that of Enola (Fig. 1). These included the following materials: (i)

Table 1. Common bean materials analyzed in this study.

Entry No.	CIAT No.	Name	Alternate designations	Country†	State‡	Source§
1¶		Enola 2000		USA		ATCC
2¶		Canario 707		USA		Steve Temple, UC Davis
3	G02400	Mantequilla	Gentry 21953; PI312090	MEX	SON	CIAT
4	G03273	Morado de Aguascalientes	AGS-74-B	MEX	AGS	CIAT
5	G03290	Flor de Mayo	AGS-88	MEX	AGS	CIAT
6	G03504	Ojo de Cabra	CHIH-31; X-15267	MEX	CHI	CIAT
7	G03715	Porrillo-1		ELS		CIAT
8	G04390	Pinto	TLAX-51	MEX	TLX	CIAT
9	G04456	Jamapa		MEX	VER	CIAT
10	G04471	Cristal Blanco		CLE		CIAT
11	G04474	Coscorrón		CLE		CIAT
12	G04666	Magdalena 3		COL	MAG	CIAT
13	G04922	Rojo de Seda		HDR		CIAT
14	G05024	Jalo	BZL-0237; collected 1935	BRA		CIAT
15	G05036	Mulatinho		BRA		CIAT
16	G05254	Bagajo		BRA		CIAT
17	G05910	Burros Grandes		CLE		CIAT
18	G06861	Bayo		HDR		CIAT
19	G07385	Uribe Redondo		COL		CIAT
20	G08159	Radical		COL		CIAT
21	G11013	Bayo		MEX	DUR	CIAT
22	G11295	Frijola	GTO-55-2; MEX-187	MEX	GTO	CIAT
23	G11511	Frutilla	CLE-027	CLE		CIAT
24	G11733	Caballero		PER		CIAT
25¶	G11891	Culiacán	CULIACAN-11-57R-M-37-M-M	MEX	SIN	CIAT
26	G12717	Bolón Rojo		COL	NAR	CIAT
27	G19068	Apetito	JAL-4; PI313367	MEX	JAL	CIAT
28¶	G13094	Mayocoba	collected 1959	MEX		CIAT
29	G20553	Conejo	NVRS-431	MEX		CIAT
30	G19646	Quqa Pava		PER	CAJ	CIAT
31	G21720	Cargabello		COL		CIAT
32	G22041	Garbancillo Zarco		MEX		CIAT
33¶	G22215	II8FR MO-5-3-M-2-1-M		MEX		CIAT
34¶	G22227	MO-85-86 2598	SIN 9	MEX	SIN	CIAT
35¶	G22230	MO-85-86 2780	SIN 12	MEX	SIN	CIAT
36	G24554	Tórtolas Corriente		CLE		CIAT
37	G50517	G50517	OT-646; Cargamanto	COL	ANT	CIAT
38¶		Woodland Yellow		USA	NEB	J. Kami
39		BAT93				CIAT
40		Jalo EEP558		BRA		CIAT
41¶		Sulphur BN142	= A	USA		J. Nienhuis and K. Kmiecik, University of Wisconsin
42¶		Mayocoba 1998		USA		PRO
43¶		Mayocoba 2001		USA		PRO
44¶		Myasi 2001		USA		PRO
45¶		Frijol Canario		PER		P. Gepts
46¶		Azufrado Peruano 87		MEX		J. Acosta (INIFAP)
47¶		Azufrado Regional 87		MEX		J. Acosta (INIFAP)
48¶		Azufrado Regional 87		MEX		INIFAP
49¶		Azufrado Peruano 87		MEX		INIFAP
50¶		Azufrado Pimono78		MEX		INIFAP
51¶		Enola 2001		USA		PRO
52¶		Enola 2000-2		USA		ATCC
53¶		Enola-PRO	= B	USA		PRO
54¶		Mayocoba	= C	USA		PRO
55¶		Myasi	= D	USA		PRO
56¶		Enola 2002	= E	USA		ATCC

† ISO country codes: BRA: Brazil; CLE: Chile; COL: Colombia; ELS: El Salvador; HDR: Honduras; MEX: Mexico; PER: Peru; USA: United States of America.

‡ State, province, or department code: AGS: Aguascalientes; ANT: Antioquia; CAJ: Cajamarca; CHI: Chihuahua; DUR: Durango; GTO: Guanajuato; JAL: Jalisco; NAR: Nayarit; NEB: Nebraska; SON: Sonora; TLX: Tlaxcala; VER: Veracruz.

§ Sources: ATCC: American Type Culture Collection; INIFAP: Instituto Nacional de Investigaciones Forestales y Agropecuarias, Mexico; PRO: Proprietary source; UC Davis: University of California, Davis.

¶ Indicates a yellow-seeded cultivar.

Table 2. Levels of polymorphism identified by *EcoRI/MseI* and *PstI/MseI* primer combinations of AFLP markers.

Primer combinations	All AFLP fragments: Total number	All AFLP fragments: Mean no. per primer pair	All AFLP fragments: Proportion of total number (%)	Polymorphic AFLP fragments: Total number	Polymorphic AFLP fragments: Frequency of polymorphism (%)	Polymorphic AFLP fragments: Proportion of total polymorphic number (%)	Assay efficiency index
EcoRI/MseI†	314	63	46	34	11	26	11
PstI/MseI†	376	75	54	99	26	74	31
Totals	690	69		133	19		21

† 5 primer combinations (see Materials and Methods).

EIGEN, and 3DPLOT programs of NTSYS (Rohlf, 1997). For the second experiment, genetic similarities expressed as Dice's coefficient were calculated using the SIMQUAL program of NTSYS. A dendrogram was then calculated on the basis of the Unweighted Paired Group Method using Arithmetic averages algorithm implemented in the SAHN program of NTSYS. Bootstrap values of the clusters were calculated on the basis of 10 000 replications with the WINBOOT program (http://www.irri.org/textonly/science/software%20downloads/winboot.htm; verified 27 January 2004).

Calculation of DNA Fingerprinting Profile Probabilities

Five possible breeding scenarios were considered to have given rise to cultivar Enola. Three of these involved hybridization between inbred (homozygous) cultivars and two involved selection within a Peruano-type cultivar (see Results section). For each of the three scenarios involving cultivar hybridizations (Table 3), the probabilities of obtaining the Enola profile were calculated as $\tilde{P} = \prod_i \tilde{p}_i^2$ (Weir 1996, p. 218), with $\tilde{p}_i$ being the probability of obtaining the ith fragment state observed for the Enola profile (either presence or absence of the fragment). This formula is valid only if AFLP fragments show independence among each other. We defined independent markers as those markers for which less than 10% of the Fisher exact tests for independence with all other markers were statistically significant ($P \leq 0.10$). For the first scenario (a cross between any Andean and Middle American cultivars):

$$\tilde{p}_i = \begin{cases} u_i^A u_i^M + 0.5 u_i^A v_i^M + 0.5 v_i^A u_i^M \\ v_i^A v_i^M + 0.5 v_i^A u_i^M + 0.5 u_i^A v_i^M \end{cases}$$

for the presence or absence of fragment i, respectively, where u_i^A, v_i^A, u_i^M, and v_i^M 0 are the frequencies of the presence (u) or absence (v) of fragment i in the Andean (A) and Mesoamerican (M) gene pools, respectively. For the second scenario [a cross between representatives of yellow-seeded cultivars of the Andean (Frijol Canario) and Middle American (Mayocoba: G13094) gene pools]:

$$p_i = \begin{cases} 1 \\ 0.5 \end{cases}$$

when both parents show the same or different fragment i state (present or absent), respectively, as the Enola profile. For the third scenario (cross between members of the group of yellow-seeded cultivars):

$$\tilde{p}_i = \begin{cases} (u_i^Y)^2 + u_i^Y v_i^Y \\ (v_i^Y)^2 + u_i^Y v_i^Y \end{cases}$$

for the presence or absence of fragment i, respectively, where u_i^Y and v_i^Y are the frequencies for the presence (u) and absence (v) of the ith fragment among yellow-seeded beans. For the fourth and fifth scenarios involving selections within existing Peruano-type cultivars, the probability of the Enola profile was calculated as $\tilde{P} = n_A/n$ 0, where n_A is the frequency of individuals with the Enola marker profile in the sample of size n of the respective cultivars. Variances for the crossing scenarios were calculated as in Weir (1996, p. 218) and those for the selection scenario as $n_A (1 - n_A)/n$. For all scenarios, homozygosity of the cultivars was assumed on the basis of the predominantly self-pollinating nature of *P. vulgaris*.

Leaf Color Analysis

Leaf color was analyzed in a greenhouse experiment, planted on 14 May 2002. Yellow-seeded entries (No. 42–52 of Table 1) were included in the experiment. Color observations were made on 4 and 5 June 2002. At those dates, plants had a fully expanded first trifoliolate and an expanding second trifoliolate. The experimental design was a randomized complete block design with three replicates. The experimental unit was a single pot with four plants. Three measurements were made on the first trifoliolate of each plant. The three measurements were then averaged and further statistical calculations were based on these averages. Leaf color measurements were conducted with a Minolta Chroma Meter CR-200 (Minolta, Ramsey, NJ), a tristimulus colorimeter, calibrated with a standard white tile (Y 94.6 x 0.3143 y 0.3209) and a standard green tile (Y 34.0 x 0.2770 y 0.3650). Results were reported in L*, chroma ($\sqrt{a^{*2} + b^{*2}}$) and hue angle (arctangent b*/a*). The value L* is a measure of lightness, it ranges from 0 (black) to 100 (white). The value L* is a measure of lightness, it ranges from 0 (black) to 100 (white). Chroma is a measure of color saturation or intensity, and hue angle denotes the color (an angle of 0° corresponds to red-purple, 90° to yellow, 180° to bluish-green and 270° to blue (McGuire, 1992).

Results for the three variables—Lightness (L), Chroma (C), and Hue Angle (h)—were first examined for a fit to a normal distribution using the PROC UNIVARIATE procedure of SAS. All three variables exhibited normality according to a Shapiro-Wilk test ($r > 0.95$). Differences among cultivars

Table 3. Probability of the AFLP marker profile shown by cultivar Enola assuming various hypothetical breeding scenarios.

Breeding scenario†	Number of independent markers	Probability	Variance
Cross between any Andean and Mesoamerican cultivar analyzed in this study	31	1×10^{-18}	2×10^{-18}
Cross between original yellow-seeded Middle American (Mayocoba, G13094) and Andean (Frijol Canario) cultivars	31	3×10^{-14}	5×10^{-12}
Cross between any pair of yellow-seeded cultivars	24	3×10^{-5}	2×10^{-4}
Selection without crossing from:			
Mayocoba	–	6×10^{-2}	4×10^{-3}
Azufrado Peruano 87	–	3×10^{-1}	1×10^{-3}

† See text for further explanations on the scenarios.

were analyzed by the PROC GLM procedure of SAS using a mixed model, with cultivars as a fixed factor and replicates as the random factor, and a Type III expected mean square. Following rank transformations, comparisons among means were conducted based on least squares means, adjusted for multiple comparisons by the Tukey-Kramer procedure.

RESULTS

Levels of Polymorphism Observed with AFLPs

In a first experiment, we used AFLP markers (Vos et al., 1995) as modified (Barcaccia et al., 1999) to determine the genetic relationships among bean cultivars. A panel of 56 bean entries was analyzed (Table 1) and consisted of 32 cultivars representative of the genetic diversity of beans in general (Singh et al., 1991a) and 24 cultivars with yellow seeds similar to those of the cultivar Enola (see Materials and Methods).

The 10 AFLP primer combinations (five *Eco*RI/*Mse*I and five *Pst*I/*Mse*I) revealed 133 polymorphic amplified fragments among 690 amplified fragments (19% polymorphism) in the sample of Andean and Mesoamerican accessions (Table 2). There was a marked difference between the *Eco*RI/*Mse*I primers and *Pst*I/*Mse*I primers in the number of polymorphic markers identified. *Pst*I/*Mse*I primers produced a slightly larger proportion of fragments compared to *Eco*RI/*Pst*I primers (54 vs. 46%). However, the frequency of polymorphic fragments was substantially higher among the former compared to the latter (26% vs. 11%). Taking into account both the total number of amplified fragments and the level of polymorphism, *Pst*I/*Mse*I primers were three-fold more powerful in detecting polymorphisms than the *Eco*RI/*Mse*I primers (76 vs. 24%, respectively, of the total number of polymorphic bands detected in this experiment; Assay Efficiency Index (Porceddu et al., 2002) of 31 vs. 11, respectively) (Table 2).

Genetic Relationships in a Representative Sample of Common Bean Cultivars

The first three coordinates of the principal coordinates analysis of AFLP markers explained 58, 7, and 5%, respectively, of the variation observed (Fig. 2). The first principal coordinate separated Middle American (negative coordinates) from Andean (positive) bean domesticates as previously observed (Gepts et al., 1986; Singh et al., 1991a). Mean genetic similarity estimates within the two groups were 0.90 (Andean gene pool) and 0.77 (Middle American gene pool), whereas between them it was 0.43, confirming the existence of a major genetic differentiation between the two gene pools. The second principal coordinate separated the Mesoamerica race (positive coordinates) from races Durango and Jalisco (negative coordinates, Fig. 2) (Singh et al., 1991a). However, a high level of genetic similarity was observed between these two subgroups (0.74). The third axis separated the yellow-seeded group (positive coordinates) of the Peruano type from the rest of the Andean cultivars (negative coordinates). Within the Mesoamerican group, a yellow-seeded cultivar (G13094) is a representative of the Mesoamerican parent of the Peruano class. It was collected in 1959 before breeding programs were initiated that led to the development of the Peruano cultivars in Mexico. A representative of the Andean parent of the Peruano class is Frijol Canario, situated as expected within the Andean group in Fig. 2.

Within the yellow-seeded Andean group, the cultivar Enola (obtained from its official source at the American Type Culture Collection) was part of a tightly knit group including the Mexican cultivar Azufrado Peruano 87 as well as the U.S. cultivar Myasi (represented by two samples, Myasi and Myasi 2001), suggesting a very close genetic relationship, if not an identity, between these cultivars (Fig. 2). Of particular importance is the comparison between Enola and Azufrado Peruano 87 given the Mexican origin of Enola as stated in the patent (Proctor, 1999) and the PVP certificate (U.S. Department of Agriculture, http://www.ars-grin.gov/cgi-bin/npgs/html/acchtml.pl?1536394; verified 8 January 2004). The individual of Enola that was tested was identical or differed by a single fragment from the 133-fragment AFLP profile of the individual from Azufrado Peruano 87 depending on the source of the latter cultivar. Additional slight differences in fingerprinting pattern among different samples of the same variety (e.g., Azufrado Regional, Mayocoba) suggested that low levels of AFLP polymorphism could be present in cultivars of common bean.

Variability within Yellow Bean Cultivars

Therefore, a second experiment was conducted to confirm the fingerprinting patterns established in the first experiment, to assess the level of intracultivar AFLP polymorphism, and further investigate the relationship between Enola and Azufrado Peruano 87. AFLP fingerprinting was conducted on a sample of 16 individuals each for three cultivars (including one individual analyzed already in the previous experiment as a positive control). In addition to Enola and Azufrado Peruano 87 (entries 1 and 49 in Table 1), a sample of the U.S. proprietary cultivar Mayocoba harvested in 1998 was also included. The number of individuals analyzed per cultivar (16) was chosen in part to run all three samples side by side on the same acrylamide gel to facilitate comparisons.

Thirty-two of the same 133 fragments observed in the first experiment were polymorphic. The Enola sample was monomorphic; all 16 individuals of Enola showing the same fragment profile (Fig. 3). The Mayocoba 1998 sample showed five different combinations of fragments among 16 individuals, with one individual each harboring the same profile as in the Enola and Azufrado Peruano 87 samples, respectively. The Azufrado Peruano 87 sample exhibited eight combinations. Five of the individuals showed the same profile as in Enola.

A cluster analysis (Rohlf, 1997) of the 48 individuals analyzed confirmed that Azufrado Peruano 87 was more closely related to Enola than Mayocoba 1998 (Fig. 3). Fourteen of the sixteen individuals of Mayocoba clustered in a group that was highly supported by a bootstrap analysis (score of 96). The sixteenth individual tested of this cultivar had the same combination of AFLP

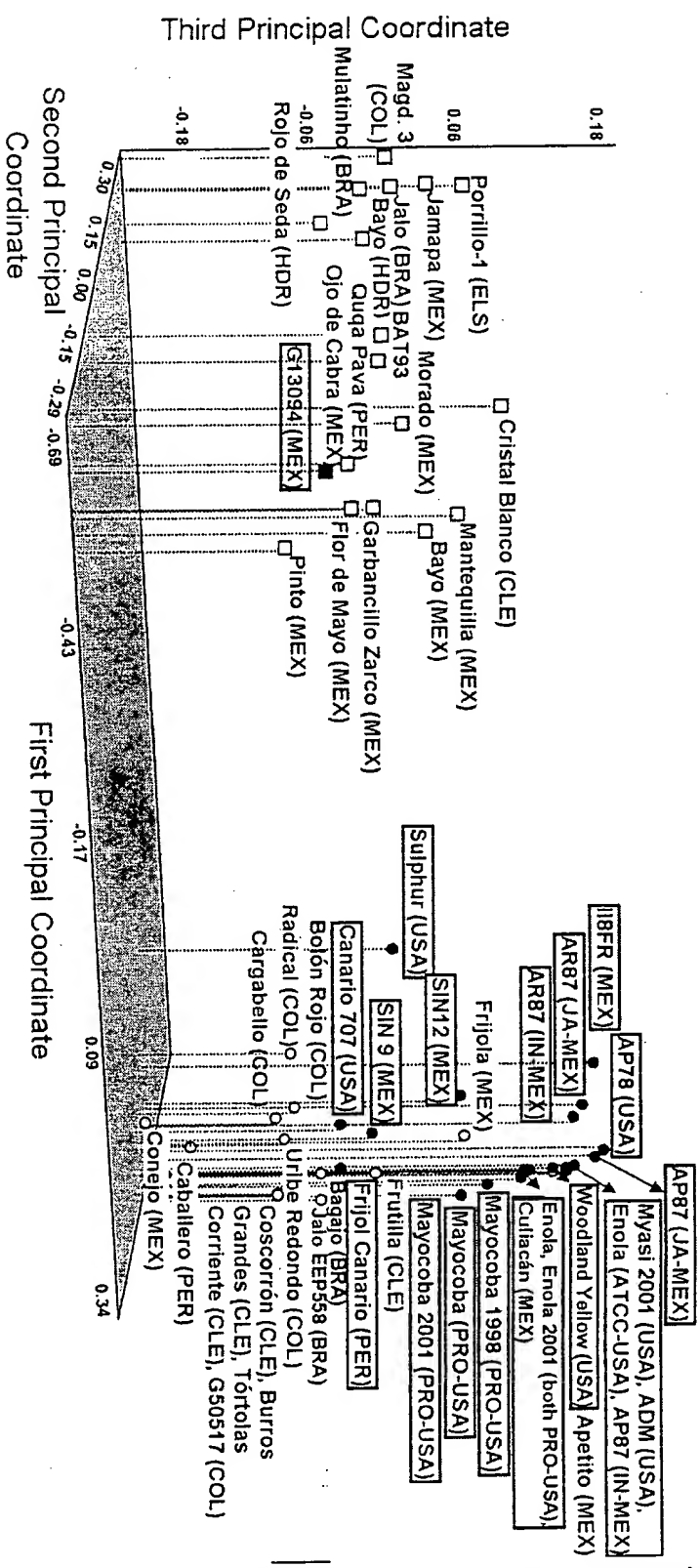


Fig. 2. Principal coordinate analysis of AFLP diversity in a sample of 56 common bean cultivars. Square symbols: Middle American gene pool; circles: Andean gene pool. Boxed entries and filled symbols: yellow seed coat entries. AP78: Azufrado Peruano 78; AP87: Azufrado Peruano 87; AR: Azufrado Regional 87. The eigenvalues of the three axes are 58, 7, and 5%. For further explanations about the identity of the different groups, see text. For the identity of each entry, see Table 1.

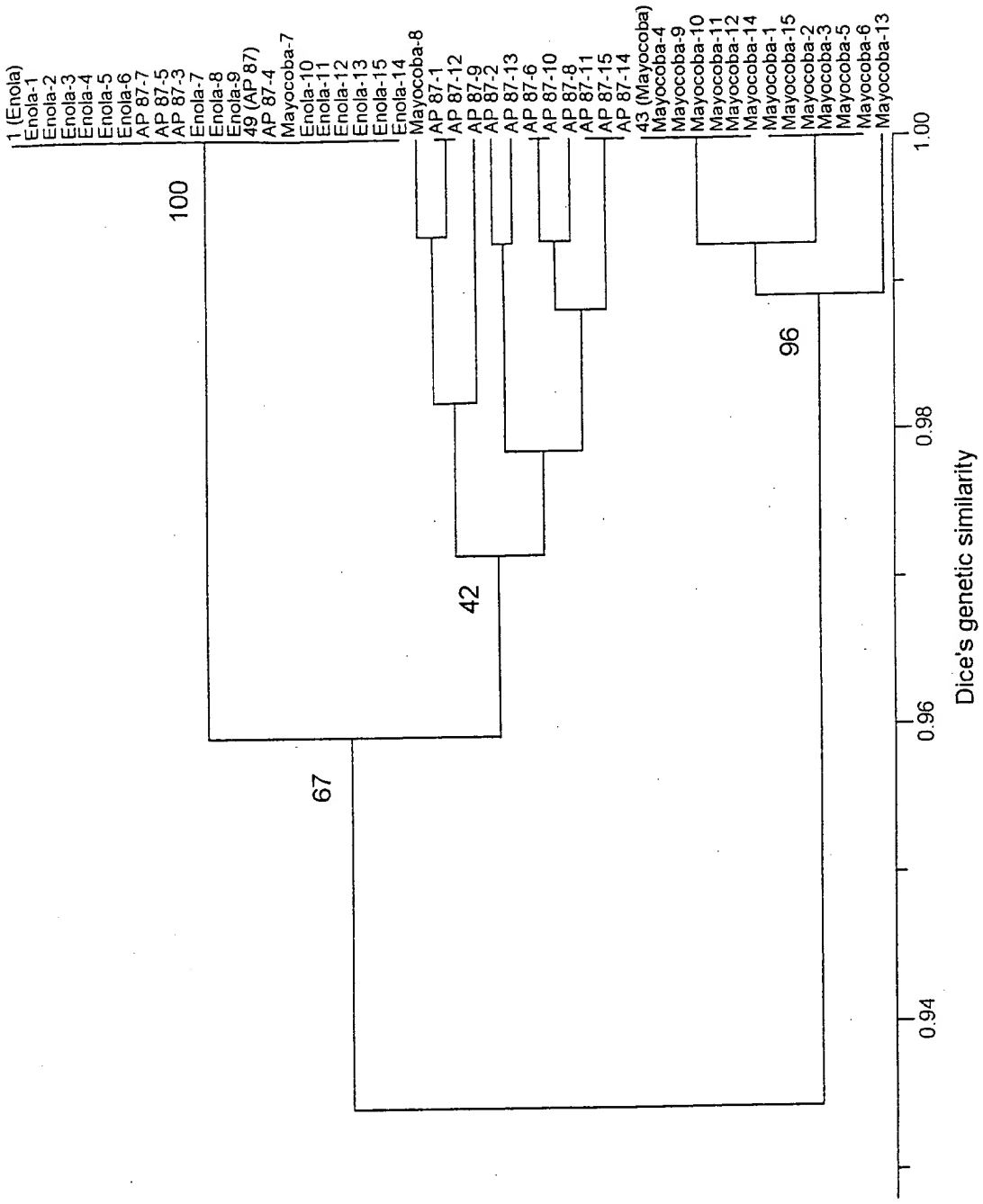


Fig. 3. UPGMA dendrogram showing the relationships among AFLP fragment profiles found in three Peruano-type bean cultivars: Enola, Azufrado Peruano 87 (AP87), and Mayocoba 1998. Each branch represents a different combination. Individuals to the right of vertical bars have identical combinations. The numbers within the tree are bootstrap values.

fragments as Enola. Cultivars Enola and Azufrado Peruano 87 formed a joint cluster with moderate to high support (score of 67). However, the poor support (score of 42) for an Azufrado Peruano 87 cluster separate from an Enola cluster suggests that the two samples are not significantly distinct. This can be attributed to the overall similarity among the different genotypes characterizing this group (Dice genetic similarity >0.96, Fig. 3), as well as the fact that the Enola profile was identical to that of five individuals of the sample of Azufrado Peruano 87.

Probabilities of Obtaining the Enola Fingerprint under Different Breeding Scenarios

Several scenarios were considered to account for the possible origin of Enola (Table 3). Three of these involved hybridization between genotypes of different evolutionary origins and two involved selection and

pure-line development within existing yellow-seeded (sub)populations. The hybridization scenarios reflect information about the origin of the Peruano commercial type, whereas the selection within yellow bean population scenarios reflected the information contained in the Enola patent and PVP certificate. Both major geographic gene pools of common bean contain yellow beans, generally called Canarios in Peru and Azufrados in Mexico. Hybridization between these two classes by Mexican bean breeder H. López, with the collaboration of F. Hernández, lead to the creation, with the release in 1978 of Azufrado Pimono 78, of a new commercial class, the Azufrados Peruanos (Voysest, 2000). This new class has a more compact growth habit and more intensely yellow seed. It is also possible, however, that yellow beans can appear by recombination between parents, one or both of which do not have yellow seeds (S. Temple, pers. comm.). The seed color genotype responsible for the yellow seed color characteristic of the Peruano types consists of seven genes. Thus, a cross between complementary genotypes at these seed color genes can also lead to yellow-seeded progeny. A third hybridization scenario reflects hybridization between two preexisting, yellow-seeded beans of the Peruano type. Both the patent and the PVP certificate describe how the Enola cultivar was developed by selection within an existing yellow bean population acquired in Mexico and grown for 3 yr in Colorado. For each of these scenarios the probability of the Enola haplotype was calculated on the basis of the frequency of the individual fragments in the populations of origin (see Materials and Methods; Table 3). Calculations were made easier because only a single fragment profile was identified for Enola (Fig. 3). As expected, the least probable scenario was the one in which the Enola fragment combination resulted from a cross between Andean and Mesoamerican genotypes (regardless of their seed color) represented in our sample (probability of 1×10^{-18}; Table 3). The scenario with the highest probability represented selection without hybridization within cultivar Azufrado Peruano 87 (3×10^{-1}). The three other scenarios had intermediate probabilities.

Comparison of Leaf Color among Yellow-Seeded Cultivars

Leaf color was examined because the statement of distinctness included in the Exhibit B (Statement of Distinctness) of the PVP certificate states that Enola most closely resembles the cultivar Azufrado Pimono 78 in a range of traits but differs from it in regards to leaf color, with Enola having lighter-colored leaves. A greenhouse experiment was conducted to compare the leaf color of a range of yellow-seeded cultivars grown under uniform conditions. There were no significant differences among replicates within cultivars. L was significant among cultivars ($P = 0.0419$), whereas Hue angle was not significant ($P = 0.3643$). There were highly significant differences among cultivars for Chroma ($P = 0.0020$). Comparisons of means showed no significant differences among cultivars for L and Hue angle. For Chroma, the only differences observed were between one of the sources of Azufrado Peruano 87 (Entry 46 in Table 1: 33.30), on the one hand, and Enola (27.35) and Azufrado Regional 87 from two sources (28.45, 28.77), on the other hand. For a second source of Azufrado Peruano 87 (Entry 49 in Table 1: 31.62), there were no significant differences with other cultivars, including Enola. Although Azufrado Pimono 78, the comparison cultivar included for distinctness in the PVP certificate, had darker green leaves (30.99) than Enola, the difference between the two cultivars was not significant in this experiment.

DISCUSSION

Choice of Markers for Fingerprinting

To conduct a fingerprinting experiment to determine the genetic relatedness among genotypes, three important elements need to be taken into account: the type of markers, the sample of genotypes, and the statistical treatment of the fingerprinting data. Fingerprinting markers should ideally have a high level of polymorphism, be numerous, and distributed throughout the genome. Microsatellite markers are the type of marker that best fits this description. However, few markers have yet been isolated and mapped in common bean (Métais et al., 2002; Yu et al., 1999, 2000; Blair et al., 2003). AFLPs (Vos et al., 1995) are an alternative to microsatellites. Among molecular markers (Jones et al., 1997; Powell et al., 1996), amplified fragment length polymorphisms (Vos et al., 1995) are advantageous because they reveal a high number of reproducible markers, thus, increasing the probability of identifying polymorphic markers even among closely related genotypes, including in common bean (Beebe et al., 2001; Tohme et al., 1996) and other crop species as well (Barcaccia et al., 1999; Caicedo et al., 1999; Coulibaly et al., 2002; Hongtrakul et al., 1997; Lombard et al., 2000; Mace et al., 1999; Mackill et al., 1996; Maughan et al., 1996; Roa et al., 1997; Xu and Sun, 2001). The low level of polymorphism of individual AFLP fragments is compensated by the large number of fragments revealed by each primer pair. In our study, each primer pair revealed around 70 fragments, of which 10 to 30% were polymorphic. This level of polymorphism is much lower than that found for *Eco*RI/*Mse*I primers in tall fescue, *Festuca arundinacea* Schreb (57%; Mian et al., 2002), the tropical tree *Pterocarpus officinalis* Jacq. (68%; Rivera-Ocasio et al., 2002), the Ethiopian cereals *Eragrostis* spp. (58%; Ayele and Nguyen, 2000), and common bean (over 90%; Tohme et al., 1996), but comparable to that observed in rice, *Oryza sativa* L. (28%; Mackill et al., 1996) and soybean, *Glycine max* (L.) Merr. (17–31%; Maughan et al., 1996). The discrepancy in polymorphism level between the results of Tohme et al. (1996) and these results may be attributed to the type of material. Tohme et al. (1996) analyzed wild beans, whereas this study was focused on domesticated beans, which have been subjected to a bottleneck of genetic diversity during and after domestication (Gepts, 1988; Gepts et al., 1986; Sonnante et al., 1994).

Most of the studies using AFLP markers use *Eco*RI and *Mse*I as restriction enzymes. In this research, both *Eco*RI/*Mse*I and *Pst*I/*Mse*I primer combinations were used on the same set of plant materials. The advantage of using *Pst*I/*Mse*I primer combinations arose mainly from a nearly 2.5-fold higher frequency of polymorphism over *Eco*RI/*Mse*I combinations. The *Eco*RI and *Pst*I enzymes sample different regions of the genome. The *Pst*I enzyme is methylation-sensitive and cuts principally in unmethylated regions of the genome, containing expressed and mainly single-copy genes. *Eco*RI, in contrast, is methylation-insensitive and cuts DNA throughout the genome. A similar observation was made for two restriction fragment length polymorphism (RFLP) clone libraries made after digestion of genomic bean DNA with *Eco*RI/*Bam*HI and *Pst*I. Digestion with the latter enzyme gave a higher frequency of single copy, polymorphic RFLP probes compared to digestion with the former enzymes (Nodari et al., 1992).

Results of the principal component analysis revealed overall patterns of genetic diversity similar to those observed previously with other markers, such as allozymes, RFLPs, and random amplified polymorphic DNA (RAPDs), and phenotypic traits (Gepts, 1998; Singh et al., 1991a). This increased our confidence that the AFLP approach would classify bean genotypes according to well-established genetic relationships. In addition, the AFLP analysis showed that Peruano-type yellow beans represent a distinct group, emphasizing their uniqueness even within the Andean gene pool.

Choice of Bean Cultivars for Analysis

The sample of common bean accessions analyzed in this study comprised two subsamples. A first subsample included a set of landrace accessions representing the six major races identified by Singh et al. (1991a). The other subsample included accessions with a yellow seed coat color similar to that of Enola (see Materials and Methods). The goal of establishing such a sample was to determine the most likely origin of Enola. This cultivar had to be compared to other yellow-seeded materials especially from Mexico as the patent description and PVP certificate both stated that this cultivar had been introduced from that country. Yellow beans from Mexico could have three possible origins: (i) the Mesoamerican gene pool, (ii) the Andean gene pool, and (iii) hybridization between these two gene pools. Furthermore, in evaluating probabilities of a match between fingerprints of yellow beans, one also needs to consider the same probabilities for more distantly related genotypes as a control.

Novelty (or the lack of it) is determined here by genetic relationships as defined by molecular markers. Previous research by Bassett et al. (2002) has shown already that the yellow color of Enola is controlled by the same gene combination present in Peruano class of Mexico. However, it is possible that, through breeding, the same yellow color could have been obtained from different parents with complementary genes for yellow seed color or with different genes or alleles for that color, altogether. Because of genetic redundancy, different gene combinations may lead to the same phenotype, leading one to erroneously infer a genetic identity. DNA fingerprinting of individual cultivars could help distinguish among these hypotheses about the origin of the yellow seed color. A close fingerprinting relationship or a complete match in the fingerprint would indicate that Enola is directly derived from Mexican yellow-seeded cultivars. In contrast, differences in fingerprinting between Enola and other cultivars would suggest a more distant relationship as would arise, for example, through breeding by hybridization.

Relationship of Current Data with the Enola Patent and PVP Certificate

Both the principal coordinate analysis of genetic diversity in our sample of common bean (Exp. 1) and the fingerprinting of the three cultivars (Enola, Azufrado Peruano 87, and Mayocoba; Exp. 2) show that Enola is most closely related to Azufrado Peruano 87 in our sample of yellow-seeded beans. Calculations of the probability of matching AFLP fingerprints showed that the most likely origin of Enola is by selection within pre-existing Mexican Peruano-type cultivars. This finding is consistent with the history of this genotype as outlined in the Enola patent and Appendix A of the PVP certificate. Both documents explain that Enola was obtained by selection of yellow seeds within a bean population heterogeneous for seed color, obtained in Mexico in 1994. Further presumed selection for yellow seed color, growth habit and height, flower and pod color, and leaf shape and size took place in 1995 and 1996 (Appendix A of the PVP Certificate). The uniformity of the AFLP banding pattern suggests that the sample submitted to the ATCC resulted from single seed selection during several generations before submission of the required seed sample to the ATCC.

In summary, we have determined the most likely origin of the cultivar Enola. The near to complete identity with pre-existing Mexican Peruano-type cultivars (present data) and the identity of the yellow seed color genotype with that of existing yellow bean cultivars (Bassett et al., 2002) raise questions about the rationales for the award of a utility patent and a PVP certificate for Enola. These questions are beyond the scope of this article.

ACKNOWLEDGMENTS

Research partially supported by the USDA-ARS (58-5348-2-806). Thanks to D. Debouck and J. Acosta for providing seeds of germplasm accessions. Discussions with J. David and statistical advice from M. Watnik are greatly appreciated.

REFERENCES

Anonymous. 2000. Situación actual y perspectiva de la producción de Frijol en México 1990- 2000. Secretaria de Agricultura y Ganadería, Servicio de Información y Estadística Agroalimentaria y Pesquera, Mexico, DF.

Ayele, M., and H.T. Nguyen. 2000. Evaluation of amplified fragment length polymorphism markers in tef, *Eragrostis tef* (Zucc.) Trotter, and related species. Plant Breed. 119:403–409.

Barcaccia, G., E. Albertini, S. Tavoletti, M. Falcinelli, and F. Veronesi. 1999. AFLP fingerprinting in *Medicago* spp.: Its development and application in linkage mapping. Plant Breed. 118:335–340.

Bassett, M.J., R. Lee, C. Otto, and P.E. McClean. 2002. Classical and molecular genetic studies of the strong greenish yellow seedcoat

color in 'Wagenaar' and 'Enola' common bean. J. Am. Soc. Hortic. Sci. 127:50–55.

Beebe, S., J. Rengifo, E. Gaitan, M.C. Duque, and J. Tohme. 2001. Diversity and origin of Andean landraces of common bean. Crop Sci. 41:854–862.

Blair, M.W., F. Pedraza, H.F. Buendia, E. Gaitán-Solís, S.E. Beebe, P. Gepts, and J. Tohme. 2003. Development of a genome-wide anchored microsatellite map for common bean (*Phaseolus vulgaris* L.). Theor. Appl. Genet. 107:1362–1374.

Caicedo, A.L., E. Gaitan, M.C. Duque, O.T. Chica, D.G. Debouck, and J. Tohme. 1999. AFLP fingerprinting of *Phaseolus lunatus* L. and related wild species from South America. Crop Sci. 39:1497–1507.

Coulibaly, S., R.S. Pasquet, R. Papa, and P. Gepts. 2002. AFLP analysis of the phenetic organization and genetic diversity of *Vigna unguiculata* L. Walp. reveals extensive gene flow between wild and domesticated types. Theor. Appl. Genet. [print] 104:358–366.

Friedland, J. 2000. As two men vie to sell yellow beans, litigation sprouts. March 20, Section A: p. 1, 10. Wall Street Journal, New York.

Gepts, P. 1998. Origin and evolution of common bean: past events and recent trends. HortScience 33:1124–1130.

Gepts, P. 1988. Phaseolin as an evolutionary marker. p. 215–241. *In* P. Gepts (ed.) Genetic resources of *Phaseolus* beans. Kluwer, Dordrecht, the Netherlands.

Gepts, P. 1984. Nutritional and evolutionary implications of phaseolin seed protein variability in common bean (*Phaseolus vulgaris* L.). Ph.D. (Diss. abstr. 8500675), University of Wisconsin, Madison.

Gepts, P., and M.T. Clegg. 1989. Genetic diversity in pearl millet (*Pennisetum glaucum* [L.] R.Br.) at the DNA sequence level. J. Hered. 80:203–208.

Gepts, P., T.C. Osborn, K. Rashka, and F.A. Bliss. 1986. Phaseolin-protein variability in wild forms and landraces of the common bean (*Phaseolus vulgaris*): Evidence for multiple centers of domestication. Econ. Bot. 40:451–468.

Hedrick, U. 1931. The vegetables of New York. I. Legumes, curcurbits, corn, alliums, asparagus. Part II. Beans. J.B. Lyon, Albany, NY.

Hongtrakul, V., G. Huestis, and S. Knapp. 1997. Amplified fragment length polymorphisms as a tool for DNA fingerprinting sunflower germplasm: Genetic diversity among oilseed inbred lines. Theor. Appl. Genet. 95:400–407.

Jones, C., K. Edwards, S. Castaglione, M. Winfield, F. Sala, C. van de Wiel, G. Bredemeijer, B. Vosman, M. Matthes, A. Daly, R. Brettschneider, P. Bettini, M. Buiatti, E. Maestri, A. Malcevschi, N. Marmiroli, R. Aert, G. Volckaert, J. Rueda, R. Linacero, A. Vazquez, and A. Karp. 1997. Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Mol. Breed. 3:381–390.

Lombard, V., C.P. Baril, P. Dubreuil, F. Blouet, and D. Zhang. 2000. Genetic relationships and fingerprinting of rapeseed cultivars by AFLP: Consequences for varietal registration. Crop Sci. 40:1417–1425.

Mace, E.S., R.N. Lester, and C.G. Gebhardt. 1999. AFLP analysis of genetic relationships among the cultivated eggplant, *Solanum melongena* L., and wild relatives (Solanaceae). Theor. Appl. Genet. 99:626–633.

Mackill, D., Z. Zhang, E. Redoña, and P. Colowit. 1996. Level of polymorphism and genetic mapping of AFLP markers in rice. Genome 39:969–977.

Maughan, P., M. Saghai-Maroof, G. Buss, and G. Huestis. 1996. Amplified fragment length polymorphism (AFLP) in soybean: Species diversity, inheritance, and near-isogenic line analysis. Theor. Appl. Genet. 93:392–401.

McGuire, R.G. 1992. Reporting of objective color measurements. HortScience 27:1254–1255.

Métais, I., B. Hamon, R. Jalouzot, and D. Peltier. 2002. Structure and level of genetic diversity in various bean types evidenced with microsatellite markers isolated from a genomic enriched library. Theor. Appl. Genet. 104:1346–1352.

Mian, M.A.R., A.A. Hopkins, and J.C. Zwonitzer. 2002. Determination of genetic diversity in tall fescue with AFLP markers. Crop Sci. 42:944–950.

Nodari, R.O., E.M.K. Koinange, J.D. Kelly, and P. Gepts. 1992. Towards an integrated linkage map of common bean. I. Development of genomic DNA probes and levels of restriction fragment length polymorphism. Theor. Appl. Genet. 84:186–192.

Porceddu, A., E. Albertini, G. Barcaccia, G. Marconi, F.B. Bertoli, and F. Veronesi. 2002. Development of an S-SAP technique based on a LTR-like sequence of *Medicago sativa* L. Mol. Genet. Genom. 267:107–114.

Powell, W., M. Morgante, C. Andre, M. Hanafey, J. Vogel, S. Tingey, and A. Rafalski. 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol. Breed. 2:225–238.

Pratt, T. 2001. Patent on small yellow bean provokes cry of biopiracy. March 20, Late edition—Final, Section F: p. 5, column 2. New York Times, New York.

Proctor, L. 1999. Field bean cultivar named enola, No. 5,894,079 [Online] http://patft.uspto.gov/netacgi/nph-Parser?Sect1 = PTO2&Sect2 = HITOFF&p = 1&u = /netahtml/search-bool.html&r = 1&f = G&l = 50&co1 = AND&d = ptxt&s1 = 5,894,079&OS = 5,894,079&RS = 5,894,079 (verified 7 January 2004).

Rivera-Ocasio, E., T.M. Aide, and W.O. McMillan. 2002. Patterns of genetic diversity and biogeographical history of the tropical wetland tree, *Pterocarpus officinalis* (Jacq.), in the Caribbean basin. Mol. Ecol. 11:675–683.

Roa, A., M. Maya, M. Duque, J. Tohme, A. Allem, and M. Bonierbale. 1997. AFLP analysis of relationships among cassava and other *Manihot* species. Theor. Appl. Genet. 95:741–750.

Rohlf, F.J. 1997. NTSYS-pc. Numerical taxonomy and multivariate analysis system. Version 2.02. Exeter Software, Setauket, New York.

Singh, S.P., P. Gepts, and D.G. Debouck. 1991a. Races of common bean (*Phaseolus vulgaris* L., Fabaceae). Econ. Bot. 45:379–396.

Singh, S.P., R. Nodari, and P. Gepts. 1991b. Genetic diversity in cultivated common bean. I. Allozymes. Crop Sci. 31:19–23.

Sonnante, G., T. Stockton, R.O. Nodari, V.L. Becerra Velásquez, and P. Gepts. 1994. Evolution of genetic diversity during the domestication of common-bean (*Phaseolus vulgaris* L.). Theor. Appl. Genet. 89:629–635.

Tohme, J., D.O. Gonzalez, S. Beebe, and M.C. Duque. 1996. AFLP analysis of gene pools of a wild bean core collection. Crop Sci. 36:1375–1384.

Tolan, S. 2001. A bean of a different color [Online]. Available by NPR News and American RadioWorks http://www.americanradioworks.org/features/food_politics/beans/index.html (verified 8 January 2004).

U.S. House of Representatives. 2002. Patents [Online] http://uscode.house.gov/title_35.htm (verified 8 January 2004).

U.S. Supreme Court. 1980. Diamond v. Chakrabarty, 447 U.S. 303 [Online] http://supct.law.cornell.edu/supct/cases/447us303.htm (verified 8 January 2004).

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau. 1995. AFLP: A new technique for DNA fingerprinting. Nucleic Acid Res. 23:4407–4414.

Voysest, V.O. 2000. Mejoramiento genético del frijol: Legado de variedades de América Latina 1930–1999 [Genetic improvement of bean: Legacy of varieties of Latin America 1930–1999] Centro Internacional de Agricultura Tropical, Cali, Colombia.

Weir, B.S. 1996. Genetic data analysis II Sinauer, Sunderland, MA.

Xu, F., and M. Sun. 2001. Comparative analysis of phylogenetic relationships of grain amaranths and their wild relatives (*Amaranthus*; Amaranthaceae) using internal transcribed spacer, amplified fragment length polymorphism, and double-primer fluorescent inter-simple sequence repeat markers. Mol. Phylogenet. Evol. 21:372–387.

Yu, K.F., S.J. Park, and V. Poysa. 1999. Abundance and variation of microsatellite DNA sequences in beans (*Phaseolus* and *Vigna*). Genome 42:27–34.

Yu, K., S. Park, V. Poysa, and P. Gepts. 2000. Integration of simple sequence repeat (SSR) markers into a molecular linkage map of common bean (*Phaseolus vulgaris* L.). J. Hered. 91:429–434.

8

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

Reissue Application No. 09/773,303 Group Art Unit: 1638

Filed: 31 January 2001 Examiner: Nelson, Amy J.

Reexamination of U.S. Patent No. 5,894,079 Confirmation No. 6243

For: FIELD BEAN CULTIVAR NAMED ENOLA

Applicant: Proctor, Larry M. Date: 25 March 2003

BOX REISSUE
Commissioner for Patents
Washington, DC 20231

DECLARATION OF LAURA L. CONLEY
37 C.F.R. §1.132

1. I have over five years of experience in the field of plant molecular biology, including research, development, and application of molecular markers to plant breeding. For more than two years I developed molecular markers and utilized molecular markers in plant breeding within the Biotechnology Department of Cargill Hybrid Seeds. I have a Master of Science degree in Biology from the California Institute of Technology and a Bachelor of Arts degree in Genetics from the University of California at Berkeley.

2. This Declaration presents, for the Examiner's consideration, various facts which may be relevant to patentability of the claimed invention. These facts include an amplified fragment length polymorphism (AFLP) study showing genetic diversity among individual plants in Enola deposited with ATCC.

3. Applicant has filed various Information Disclosure Statements, and among the Disclosure materials submitted is the Expert Report of Paul Gepts, Ph.D related to Civil Action No. 01-WY-2310-AJ (BNB). The Gepts Report is attached as Exhibit A to this Declaration. Dr. Gepts was used as an Expert Witness for an opposing party in litigation. Supportive data for Exhibit A is

attached as Exhibit B, and is also referred to herein as including pages DEF 001740 - DEF 001749.

4. I have read Exhibits A and B. I have also read the following article cited in the Report: P. Vos, R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, and M. Kuiper (1995) Nucl. Acids. Res. 23: 4407-4414 "AFLP: a new technique for DNA fingerprinting," which is attached as Exhibit C.

5. Exhibit B includes supporting data for Exhibit A and was produced in litigaton discovery. The present Declaration is based on my analysis of Exhibit A, together with the supplemented raw data of Exhibit B. Two separate experiments are described in the Gepts Report. Exhibit B provides data from Experiments 1 and 2. Within Exhibit B, DEF 001741 - DEF 001745 describe data from Experiment 1, and DEF 001746 - DEF 001749 describe data from Experiment 2.

6. It is unclear from Exibit A alone, which lacks the data presented in Exhibit B, what methods are used to perform the described experiments; the description of the methods is incomplete and the methods as described are substantially different from the methods in the cited reference describing the AFLP technique (see Exhibit C as noted in paragraph 4 above).

7 The AFLP technique produces patterns of DNA fragments, which are visible as bands in an electrophoresis gel. For an individual, an AFLP fragment may be present or absent. A fragment that is present is typically scored as a "1," and a fragment that is absent is typically scored as a "0." Data that is missing is typically scored as a "9." When the patterns of fragments produced for two individuals are the same, the individuals are considered to have the same haplotype. When the patterns of fragments produced for two individuals are different, the individuals are considered to have different haplotypes.

8. AFLP fragments are typically tested on each individual in an experiment. In Experiment 1, 151 AFLP fragments are scored, and in Experiment 2, 133 AFLP fragments are scored. Neither Exhibit A nor Exhibit B provides a cross-reference describing the relationship between any of the fragments scored in Experiments 1 and 2 or a rationale as to why fewer fragments are scored in Experiment 2 than in Experiment 1.

9. Without a detailed description of the methods utilized to generate the data in Exhibits A and B, and without information addressing any relationship between the fragments scored in

Experiments 1 and 2, it is not possible to determine the cause of what appear to be inconsistencies between the results of Experiments 1 and 2.

10. Exhibit A (Exhibit 2, Table 1 thereof) indicates that in Experiment 1, individual 1 is a first Enola 2000-1 seed from ATCC, and that individual 52 is a second Enola 2000-2 seed from ATCC.

11. Exhibits A and pages DEF 001741 - DEF 001745 of Exhibit B describe data on 103 AFLP fragments (151 total - 48 fragments for which data is missing) for both individuals 1 and 52.

12. Exhibit A and pages DEF 001741 - DEF 001745 of Exhibit B describe six AFLP fragments (3, 6, 20, 23, 36, and 86) that are present and/or absent in different patterns when comparing Enola individuals 1 and 52 (see Table 1 of the present Declaration).

13. The results of these six AFLP fragments (3, 6, 20, 23, 36, and 86) for Enola individuals 1 and 52 demonstrate molecular heterogeneity in Enola deposited with ATCC. These results demonstrate that the haplotype of individual 1 is different from the haplotype of individual 52. These results demonstrate that there are at least two haplotypes within Enola deposited with ATCC.

14. In Exhibit A (Exhibit 2, Table 1 thereof) and Exhibit B pages DEF 001741 - DEF 001745, individual 51 is Enola 2001 from Northern Feed and Bean (NF&B), and individual 53 (alternately designated B) is Enola-NFB from NF&B.

15. Experiments in Exhibit A and Exhibit B pages DEF 001741 - DEF 001745 describe the analysis of 151 AFLP fragments, of which fourteen AFLP fragments (3, 6, 20, 23, 36, 50, 67, 68, 86, 110, 129, 131, 145, and 151, see Table 1 of the present Declaration) are present and/or absent in different patterns when comparing at least two of the Enola individuals 1, 51, 52, and 53.

Table 1[a, b]

AFLP fragment (Expt. 1)	1	52	51	53 (B)
	Enola 2000-1 ATCC	Enola 2000-2 ATCC	Enola 2001 NF&B	Enola-NFB NF&B
3	1	0	1	1
6	1	0	1	1
20	0	1	0	0
23	0	1	0	1
36	1	0	1	0
50	0	9	0	1
67	1	9	0	1
68	0	9	1	0
86	0	1	1	0
110	0	9	0	1
129	0	9	1	1
131	1	1	0	1
145	1	1	1	0
151	0	0	0	1

[a] A "1" indicates the presence of the fragment, a "0" indicates the absence of the fragment, and "9" indicates that no data is available.

[b] Table 1 is a subset of data presented in Experiment 1 of Exhibits A and B , as collated by myself.

16. The fourteen AFLP fragments described in Table 1 can be seen to be heterogeneous among Enola individuals 1, 51, 52, and 53. Furthermore, each of these four Enola individuals has a unique pattern of these fourteen AFLP fragments relative to each other. These data demonstrate the presence of at least four haplotypes within Enola from all sources.

17. Experiment 2 of Exhibit A describes a study of sixteen Enola individuals from ATCC (Exhibit A, page 18). Data from Experiment 2 (Exhibit B pages DEF 001746 - DEF 001749) is used to describe a single haplotype of Enola designated in Exhibit A as Haplotype A (Exhibit 8, Table 2 thereof).

18. It is also unclear from Exhibit A whether any of the sixteen Enola individuals tested in Experiment 2 were tested in Experiment 1. The labels used to identify samples in Experiment 2 (see Exhibit A page 18 and Exhibit B pages DEF 001746 - DEF 001749) match labels used for samples in Experiment 1 (Exhibit A, as shown in Exhibit 2, Table 1 thereof) for Mayacoba (#43 - 2001 sample of NF&B) and for Azufrado Peruano 87 (#49 - Azufrado Peruano 87 MPP), but

not for Enola (#1 - "sample of ATCC, acquired in 2002" in Exhibit A page 18 versus "Enola 2000-1" and "ATCC" in Exhibit 8, Table 2 of Exhibit A). The description of the Enola individuals in Experiment 2 (Exhibit A page 18, "sample of ATCC, acquired in 2002") is most similar to the description of individual 56 in Experiment 1 (Exhibit A as shown in Exhibit 2, Table 1 thereof, "Enola 2002" from "ATCC"). It is irrelevant, however, whether individual 56 in Experiment 1 is the same as one of or from the same source as the sixteen individuals tested in Experiment 2, because no data is presented for individual 56 in Exhibit A (for example, see Exhibits 4-6) or Exhibit B pages DEF 001741 - DEF 001745.

19. Exhibit A does not provide a cross-reference of the fragments scored in Experiments 1 and 2, and Exhibit A does not provide data on any Enola individual common to both Experiments 1 and 2. Therefore it is not possible to determine if Haplotype A (as defined by Exhibit A) is or is not identical to one of the Enola haplotypes described in Experiment 1. Haplotype A may be an additional haplotype of Enola.

20. Despite that Experiment 2 suggests that all Enola individuals from ATCC have the same haplotype (labeled Haplotype A in Experiment 2 of Exhibit A), Experiment 1 describes the presence of at least two haplotypes in Enola from ATCC (individuals 1 and 52).

21. Exhibits A and B describe data for a total of eighteen (two in Experiment 1 and sixteen in Experiment 2) Enola individuals from ATCC. Data from Experiments 1 and 2, when combined, describe two or possibly three haplotypes in Enola deposited with ATCC. Experiments 1 and 2 describe data for a total of twenty (four in Experiment 1 and sixteen in Experiment 2) Enola individuals from all sources. The data in Experiments 1 and 2 suggest four or possibly five haplotypes within Enola from all sources combined.

22. Exhibit A and Exhibit B pages DEF 001740 - DEF 001749 demonstrate that Enola is genetically diverse in that Enola contains at least four distinct haplotypes.

23. The identification of a set of AFLP fragments that is identical for two individuals, does not "prove" homogeneity of those individuals. However, the identification of only one AFLP fragment that is different between two individuals does prove the heterogeneity of those individuals. Consequently, Experiment 2 (Exhibit A and Exhibit B pages DEF 001746 - DEF 001749) does not "prove" that the sixteen Enola individuals tested in Experiment 2 are identical or that Enola is homogeneous. The results of Experiment 2 only show that those sixteen Enola individuals are identical for the 133 markers tested in Experiment 2. The results of Experiment 1

(Exhibit A and Exhibit B pages DEF 001741 - DEF 001745), however, do demonstrate AFLP fragments that score differently for Enola individuals (Table 1 of the present Declaration). The results of Experiment 1 do prove the heterogeneity of Enola, at least at the DNA sequence level. The results of Experiment 2 do not disprove the heterogeneity of Enola established by Experiment 1.

24. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Laura L Conley
Laura L. Conley M.S.

3/25/03
Date

UNIVERSITY OF CALIFORNIA, DAVIS

BERKELEY • DAVIS • IRVINE • LOS ANGELES • RIVERSIDE • SAN DIEGO • SAN FRANCISCO

SANTA BARBARA • SANTA CRUZ

DEPARTMENT OF AGRONOMY AND RANGE SCIENCE
UNIVERSITY OF CALIFORNIA
1 SHIELDS AVENUE
DAVIS, CALIFORNIA 95616-8515
http://agronomy.ucdavis.edu/gepts/geptslab.htm

Phone: (530) 752-7743
FAX: (530) 752-4361
plgepts@ucdavis.edu

October 21, 2002

Ms. J. PIETRINI, Esq
Manatt, Phelps, and Phillips, LLP
11355 West Olympic Boulevard
Los Angeles, CA 90064-1614

Dear Ms. Pietrini,

Per your request, I am sending you the data sets for experiments 1 and 2 conducted in my laboratory in relation to the analyses of yellow-seeded bean cultivars.

For experiment 1 data, the yellow-colored columns correspond to yellow-seed entries in the experiment. For the identity of the materials, one should consult Exhibit 2, Table 1 of my report. AFLP fragments scored in experiment 1 are labeled consecutively from 1 to 151 in the leftmost column.

For experiment 2 data, the three colors correspond to the three varieties tested (Enola ATCC, Mayocoba NF&B, and Azufrado Peruano 87). The color coding is the same as in Exhibit 7, Figure 5 of my report. The leftmost column includes the consecutive numbering (from 1 to 133) of the AFLP fragments scored in this experiment. For both experiments, a value of 1 indicates the presence of the fragment and that of a 0 its absence in an entry.

Please feel free to contact me for additional information if necessary.

Yours truly,

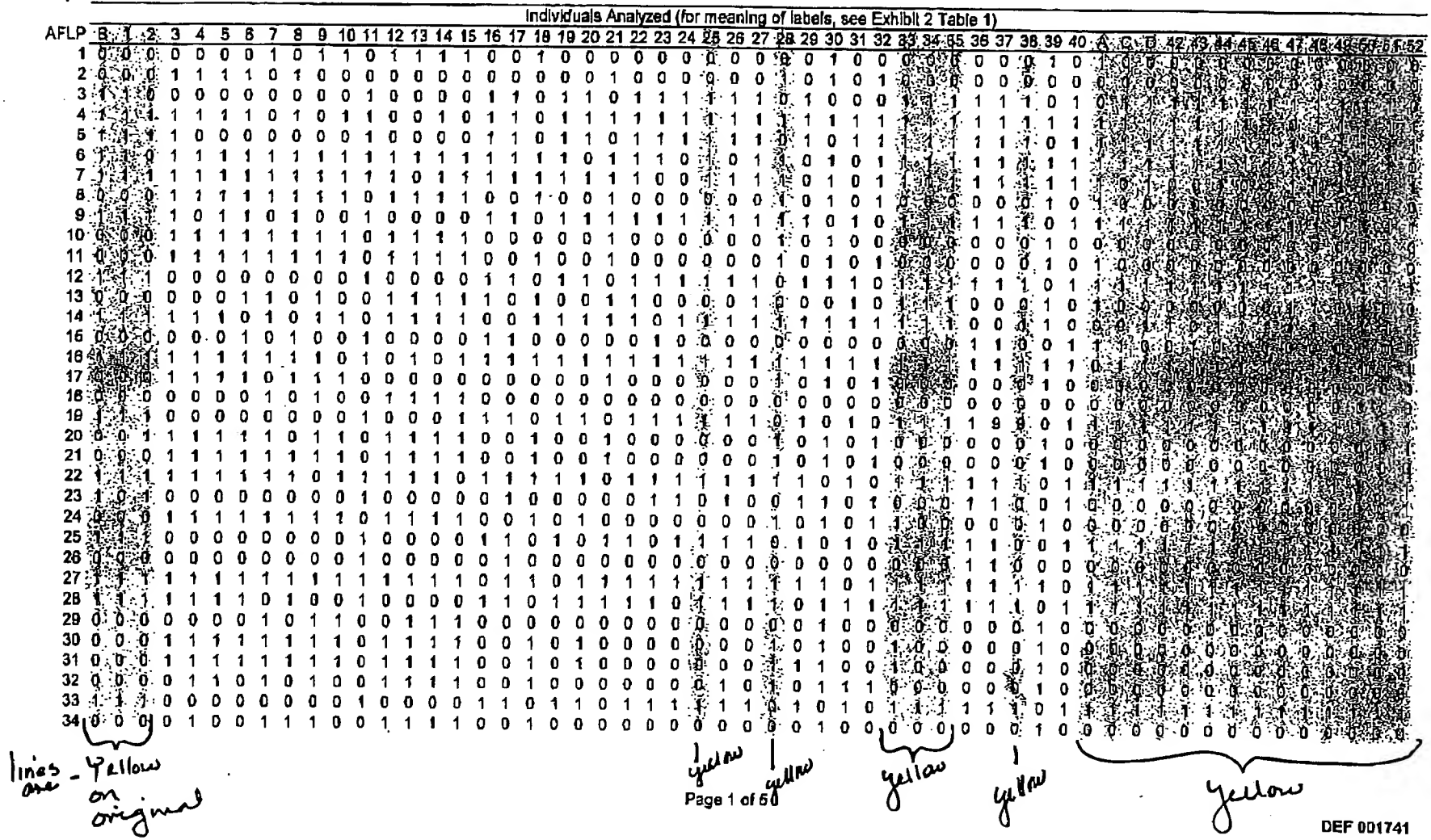
Paul Gepts
Professor of Agronomy

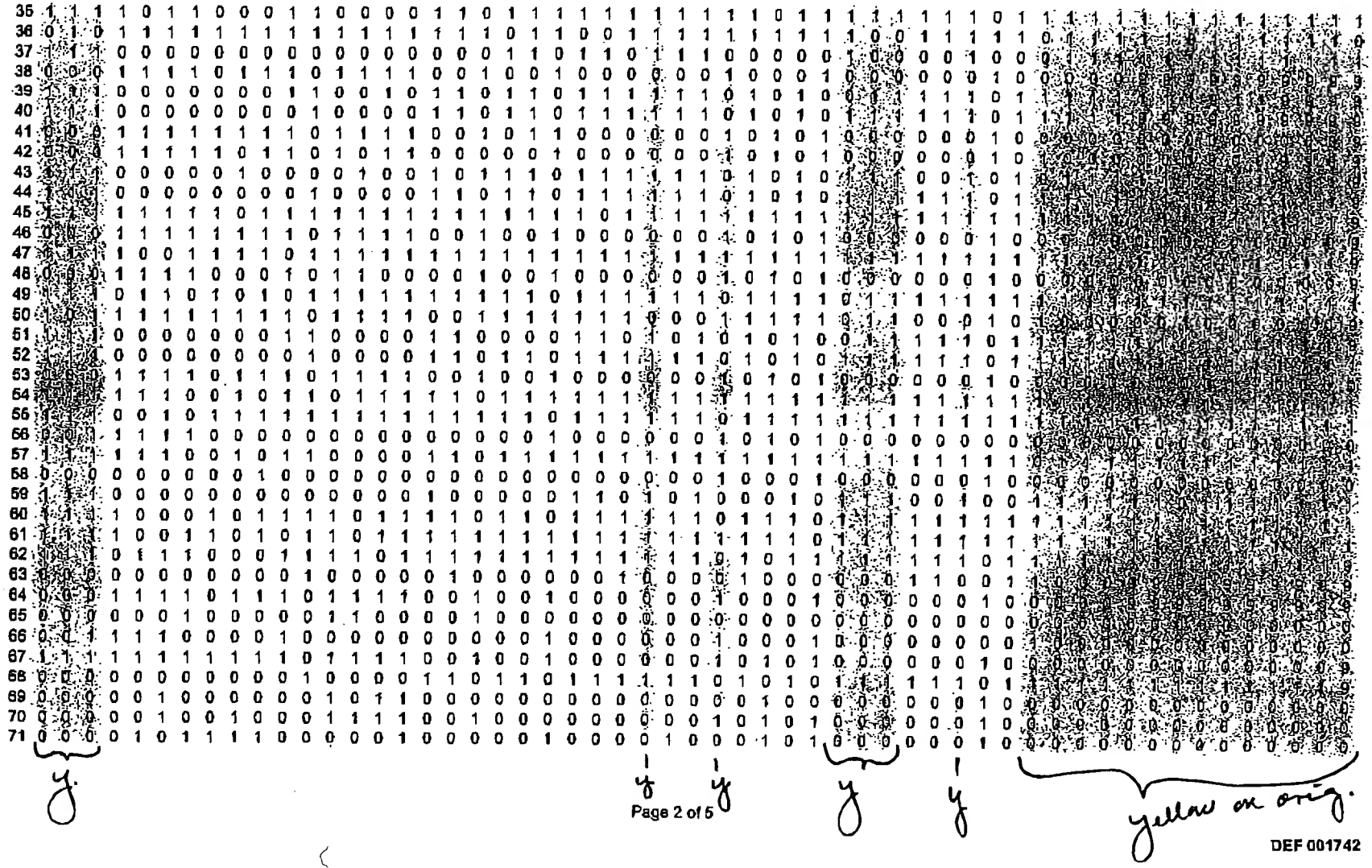
DEF 001740

7

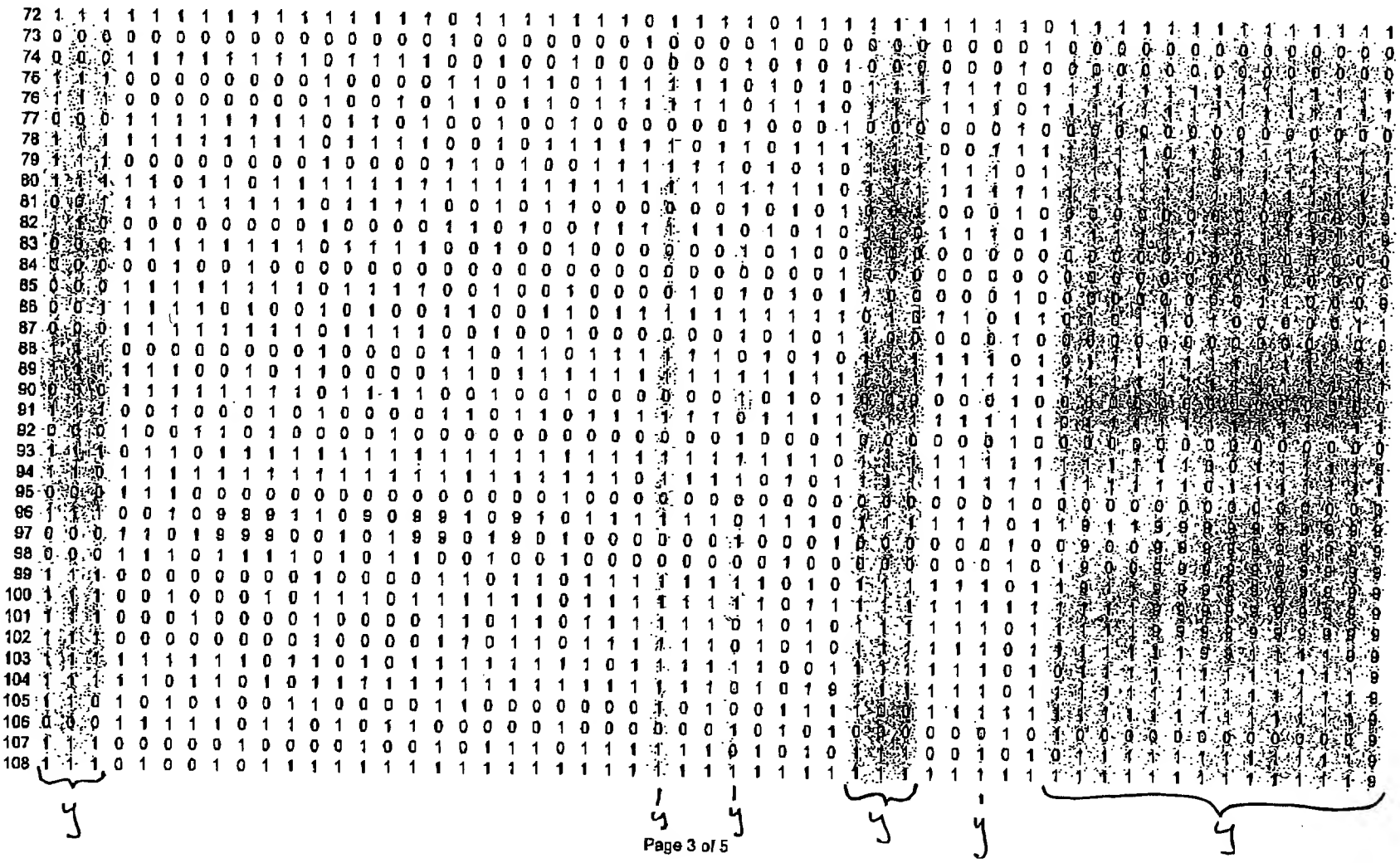
B

Experiment 1





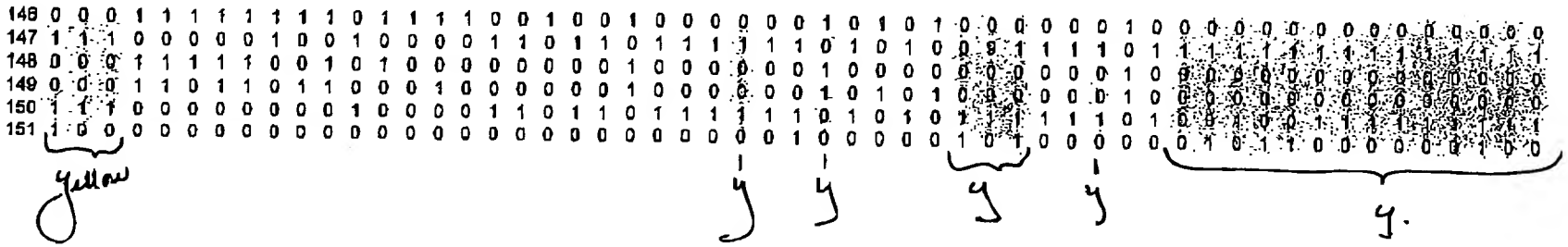
DEF 001742



109	0	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	1	0	0	1	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9
110	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	9
111	0	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	1	0	0	1	0	0	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9
112	0	0	0	1	1	0	0	1	0	1	1	0	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
113	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
114	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	1	1	0	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
115	1	1	1	0	1	1	0	0	0	0	0	1	0	0	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
116	1	1	0	0	0	0	0	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0	1	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
117	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	1	8	1	1	1	1	1	1	9
118	1	1	1	0	0	1	0	0	1	0	0	1	0	0	0	0	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
119	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
120	0	0	0	0	0	1	0	0	1	0	0	0	1	1	1	1	0	0	1	1	1	0	0	0	1	0	1	0	1	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
121	0	0	0	1	1	1	8	9	1	1	1	0	1	1	1	1	0	1	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	9	9	9	9	9	9	9	9	9	9
122	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	1	0	9	9	9	8	9	9	9	9	9	9
123	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	8	0	0	0	0	0	0	0	0	0	9	9	9	8	9	9	8	8	9	9
124	1	1	1	0	0	0	1	0	1	0	0	1	0	0	0	0	1	1	0	1	1	0	1	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	9	9	9	8	9	9	9	9	9	9
125	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	1	1	1	0	0	1	0	0	0	0	1	0	9	9	9	8	9	9	8	9	8	9
126	0	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	1	0	0	1	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9
127	1	1	1	1	0	0	0	0	0	0	1	1	0	0	0	0	1	1	0	1	1	0	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
128	0	0	0	1	1	1	1	1	0	0	1	0	1	1	1	1	0	0	1	0	1	1	0	0	0	0	0	0	1	0	1	0	1	0	0	8	0	0	0	1	0	1	0	0	0	0	0	8	0	0	0	0	0	0	0
129	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	1	1	0	0	0	1	1	1	1	1	1	0	1	0	1	0	0	1	1	1	0	1	0	0	0	0	0	0	1	0	1	1	0	1	1	9
130	1	1	1	0	0	0	0	1	0	1	0	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
131	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	1	1	0	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	0	0	0	0	0	0	1
132	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
133	0	0	0	1	0	1	0	1	0	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
134	1	1	1	1	0	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
135	0	0	0	1	1	1	1	1	0	1	1	0	1	1	1	1	0	0	1	0	0	1	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
136	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	1	1	0	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	0	1	0	0	1	0	0	1	1	1	1	1	1	1	1	1
137	0	0	0	1	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
138	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
139	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	1	1	0	1	1	1	1	1	1	0	1	0	1	0	1	0	1	1	1	1	0	1	1	1	1	1	1	1	9	8	9	9	9	8	9	9
140	1	1	1	1	1	1	1	0	0	1	0	1	1	0	0	1	1	1	0	0	0	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	9	1	1	1	1	1	1
141	0	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	1	0	0	1	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
142	1	1	1	1	1	1	1	0	1	0	1	1	0	0	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
143	1	1	1	1	0	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	0	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
144	0	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	0	1	0	0	0	0	0	0	1	0	1	0	1	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
145	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	0	0	1	1	1	1	1	1	0	1	1

DEF 001744

12

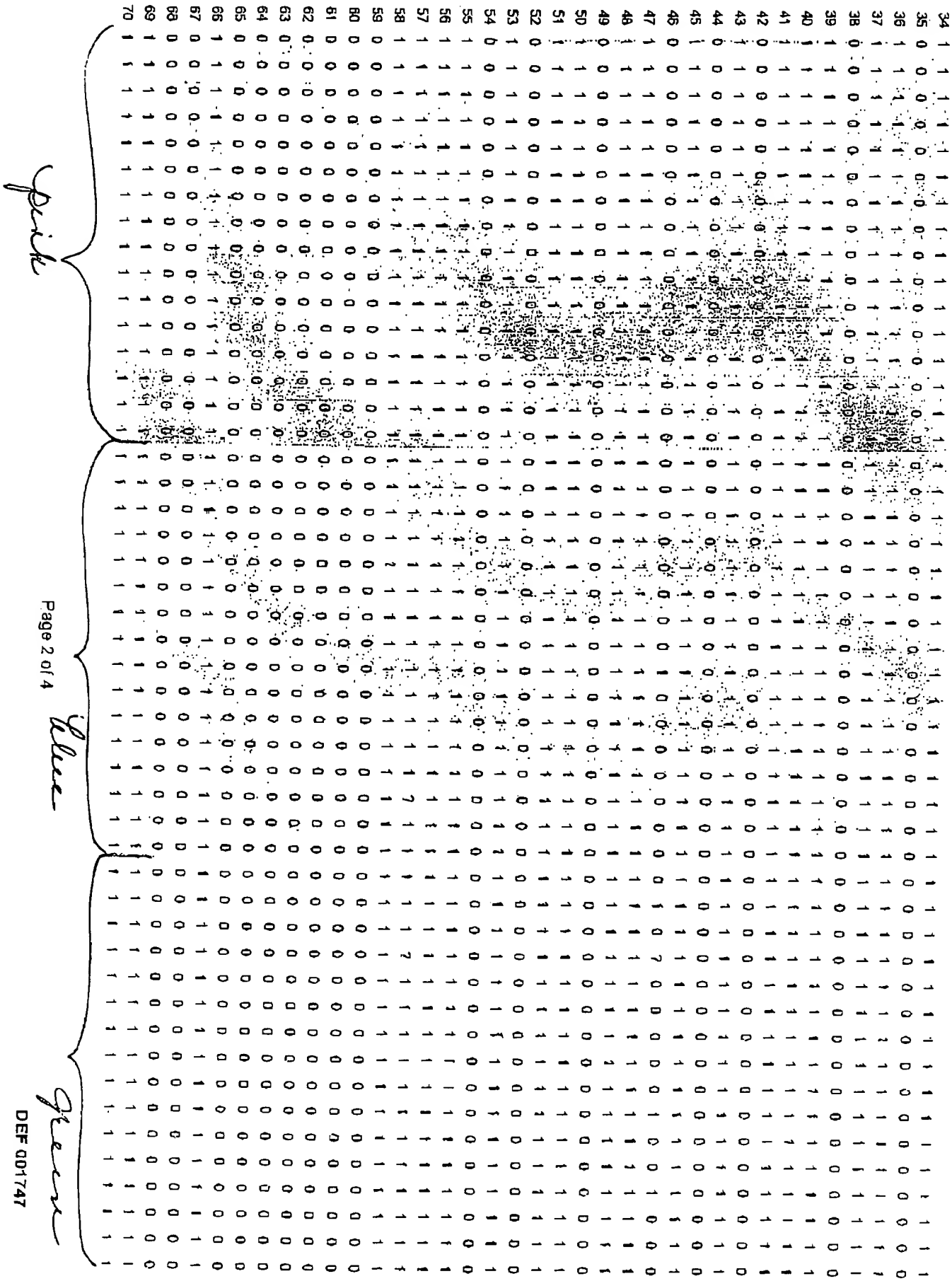


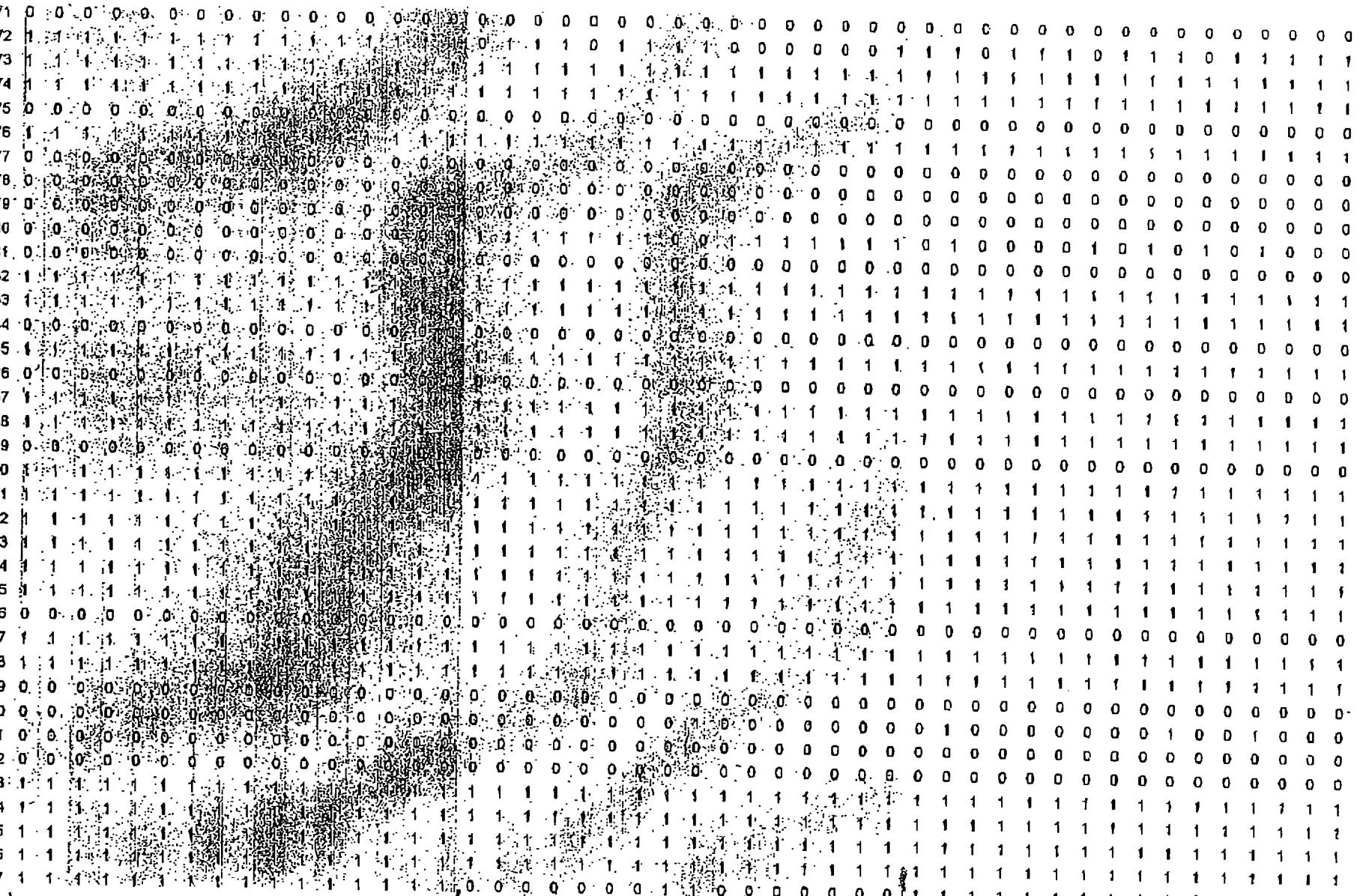
Experiment 2

Individuals Analyzed

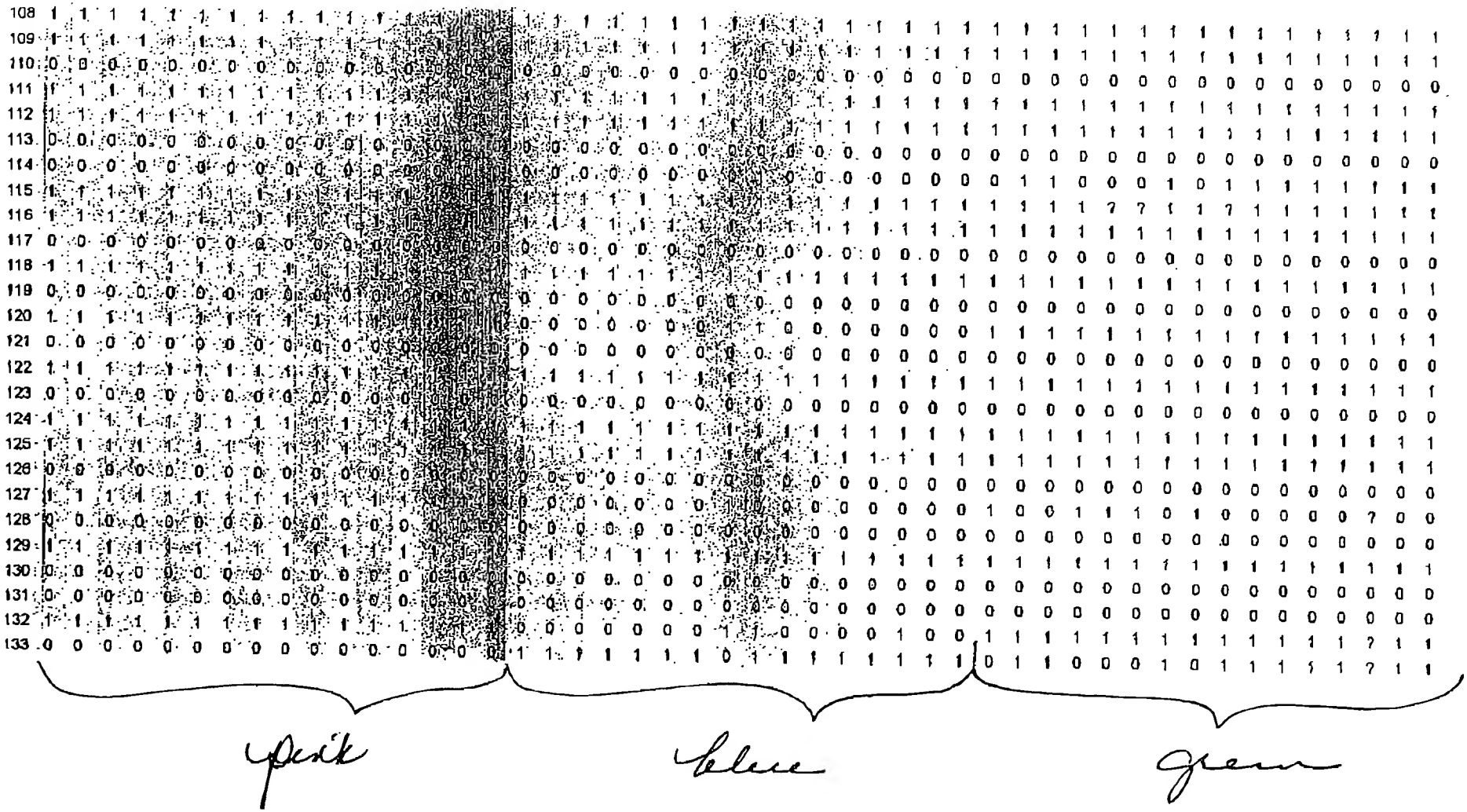
AFLP	Eriola																Mayocoba NF&B																Azufrado Peruano 87															
	1	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13	E14	E15	43	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15	49	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1
15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	1	1
16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
22	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
25	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
32	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
33	1	1	1	1	1	1	1	1	1	1	1	1	1	?	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1	?	0	0	0	0	0	0	0	0

pink Page 1 of 4 blue green





Pink Yellow Green



Nucleic Acids Research, 1995, Vol. 23, No. 21 4407–4414

AFLP: a new technique for DNA fingerprinting

Pieter Vos*, Rene Hogers, Marjo Bleeker, Martin Reijans, Theo van de Lee, Miranda Hornes, Adrie Frijters, Jerina Pot, Johan Peleman, Martin Kuiper and Marc Zabeau

Keygene N.V., PO Box 216, Wageningen, The Netherlands

Received July 14, 1995; Revised and Accepted October 5, 1995

ABSTRACT

A novel DNA fingerprinting technique called AFLP is described. The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The technique involves three steps: (i) restriction of the DNA and ligation of oligonucleotide adapters, (ii) selective amplification of sets of restriction fragments, and (iii) gel analysis of the amplified fragments. PCR amplification of restriction fragments is achieved by using the adapter and restriction site sequence as target sites for primer annealing. The selective amplification is achieved by the use of primers that extend into the restriction fragments, amplifying only those fragments in which the primer extensions match the nucleotides flanking the restriction sites. Using this method, sets of restriction fragments may be visualized by PCR without knowledge of nucleotide sequence. The method allows the specific co-amplification of high numbers of restriction fragments. The number of fragments that can be analyzed simultaneously, however, is dependent on the resolution of the detection system. Typically 50–100 restriction fragments are amplified and detected on denaturing polyacrylamide gels. The AFLP technique provides a novel and very powerful DNA fingerprinting technique for DNAs of any origin or complexity.

INTRODUCTION

DNA fingerprinting involves the display of a set of DNA fragments from a specific DNA sample. A variety of DNA fingerprinting techniques is presently available (1–11), most of which use PCR for detection of fragments. The choice of which fingerprinting technique to use, is dependent on the application e.g. DNA typing, DNA marker mapping and the organism under investigation e.g. prokaryotes, plants, animals, humans. Ideally, a fingerprinting technique should require no prior investments in terms of sequence analysis, primer synthesis or characterization of DNA probes. A number of fingerprinting methods which meet these requirements have been developed over the past few years, including random amplified polymorphic DNA (RAPD; 8), DNA amplification fingerprinting (DAF; 9) and arbitrarily primed PCR (AP-PCR; 10,11). These methods are all based on the amplification of random genomic DNA fragments by arbitrarily selected PCR primers. DNA fragment patterns may be generated of any DNA without prior sequence knowledge. The patterns generated depend on the sequence of the PCR primers and the nature of the template DNA. PCR is performed at low annealing temperatures to allow the primers to anneal to multiple loci on the DNA. DNA fragments are generated when primer binding sites are within a distance that allows amplification. In principle, a single primer is sufficient for generating band patterns. These new PCR based fingerprinting methods have the major disadvantage that they are very sensitive to the reaction conditions, DNA quality and PCR temperature profiles (12–16), which limits their application.

This paper describes a new technique for DNA fingerprinting, named AFLP. The AFLP technique is based on the detection of genomic restriction fragments by PCR amplification, and can be used for DNAs of any origin or complexity. Fingerprints are produced without prior sequence knowledge using a limited set of generic primers. The number of fragments detected in a single reaction can be 'tuned' by selection of specific primer sets. The AFLP technique is robust and reliable because stringent reaction conditions are used for primer annealing: the reliability of the RFLP technique (17,18) is combined with the power of the PCR technique (19–21). This paper describes several features of the AFLP technique and illustrates how the technique can best be used in fingerprinting of genomic DNAs.

MATERIALS AND METHODS

DNAs, enzymes and materials

Lambda DNA was purchased from Pharmacia (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). *Autographa californica* Nuclear Polyhedrosis Virus DNA (AcNPV) was a kind gift from Dr Just Vlak, Department of Virology, Agricultural University of Wageningen, The Netherlands, and was isolated as described previously (22). Acinetobacter DNA was a kind gift from Dr Paul Jansen, Department of Microbiology, University of Gent, Belgium, and was isolated from strain LMG 10554 according to the procedure of Pitcher *et al.* (23). Yeast DNA was isolated from strain AB1380 as described by Green and Olson with minor modifications (24). Tomato DNA (culture variety [cv] Moneymaker, obtained from Dr Maarten Koornneef, University of Wageningen, The Netherlands), Arabidopsis DNA (Recombinant Inbred Line 240, obtained from Dr Caroline Dean, John Innes Center, Norwich, UK), maize DNA (strain B73, obtained from Dr Mario Motto, Instituto Sperimentale per La, Bergamo, Italy), cucumber DNA (cv Primera, obtained from De Ruiter

* To whom correspondence should be addressed

17
C

Seeds C.V., Bleiswijk, The Netherlands), barley DNA (cv Ingrid, obtained from Dr Paul Schulze-Lefert, University of Aachen, Germany), lettuce DNA (cv Calmar, obtained from Dr Richard Michelmore, UC Davis, Davis, CA, USA) and brassica DNA (oil seed rape, cv Major, obtained from Dr Thomas Osborn, University of Wisconsin, Madison, WI, USA) were isolated using a modified CTAB procedure described by Stewart and Via (25). Human DNA was prepared as described by Miller *et al.* (26) from a 100 ml blood sample of Mrs Marjo Bleeker, one of the co-authors of this paper. All restriction enzymes were purchased from Pharmacia (Pharmacia LKB Biotechnology AB, Uppsala, Sweden), except for the restriction enzyme *Mse*I, which was purchased from New England Biolabs Inc. (Beverly, MA, USA). T4 DNA ligase and T4 polynucleotide kinase were also obtained from Pharmacia (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). All PCR reagents and consumables were obtained from Perkin Elmer Corp. (Norwalk, CT, USA). All radioactive reagents were purchased from Amersham (Amersham International plc, Little Chalfont, Buckinghamshire, UK) or Isotopchim (Isotopchim SA, Ganagobie, France).

AFLP primers and adapters

All oligonucleotides were made on a Biotronic Synostat D DNA-synthesizer (Eppendorf Gmbh, Maintal, Germany) or Milligen Expedite 8909 DNA-synthesizer (Millipore Corp, Bedford, MA, USA). The quality of the crude oligonucleotides was determined by end-labeling with polynucleotide kinase and [γ-^{32}P]ATP and subsequent electrophoresis on 18% denaturing polyacrylamide gels (27). Oligonucleotides were generally used as adapters and primers for AFLP analysis without further purification.

AFLP adapters consist of a core sequence and an enzyme-specific sequence (28). The structure of the *Eco*RI-adapter is:

```
5-CTCGTAGACTGCGTACC
     CATCTGACGCATGGTTAA-5
```

The structure of the *Mse*I-adapter is:

```
5-GACGATGAGTCCTGAG
      TACTCAGGACTCAT-5
```

Adapters for other 'rare cutter' enzymes were identical to the *Eco*RI-adapter with the exception that cohesive ends were used, which are compatible with these other enzymes. The *Taq*I-adapter was identical to the *Mse*I-adapter with the exception that a cohesive end was used compatible with *Taq*I.

AFLP primers consist of three parts, a core sequence, an enzyme specific sequence (ENZ) and a selective extension (EXT) (28). This is illustrated below for *Eco*RI- and *Mse*I-primers with three selective nucleotides (selective nucleotides shown as NNN):

	CORE	ENZ	EXT
*Eco*RI	5-GACTGCGTACC	AATTC	NNN-3
*Mse*I	5-GATGAGTCCTGAG	TAA	NNN-3

AFLP-primers for other 'rare cutter' enzymes were similar to the *Eco*RI-primers, and *Taq*I-primers were similar to the *Mse*I-primers, but have enzyme-specific parts corresponding to the respective enzymes.

Modification of DNA and template preparation

The protocol below describes the generation of templates for AFLP reactions using the enzyme combination *Eco*RI/*Mse*I. DNA templates with other restriction enzymes were prepared using essentially the same protocol, except for the use of different restriction enzymes and corresponding double-stranded adapters.

Genomic DNA (0.5 µg) was incubated for 1 h at 37°C with 5 U *Eco*RI and 5 U *Mse*I in 40 µl 10 mM Tris–HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 50 ng/µl BSA. Next, 10 µl of a solution containing 5 pMol *Eco*RI-adapters, 50 pMol *Mse*I-adapters, 1 U T4 DNA-ligase, 1 mM ATP in 10 mM Tris–HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 50 ng/µl BSA was added, and the incubation was continued for 3 h at 37°C. Adapters were prepared by adding equimolar amounts of both strands; adapters were not phosphorylated. After ligation, the reaction mixture was diluted to 500 µl with 10 mM Tris–HCl, 0.1 mM EDTA pH 8.0, and stored at –20°C.

AFLP reactions

Amplification reactions are described using DNA templates for the enzyme combination *Eco*RI/*Mse*I. AFLP fingerprints with other enzyme combinations were performed with appropriate primers.

AFLP reactions generally employed two oligonucleotide primers, one corresponding to the *Eco*RI-ends and one corresponding to the *Mse*I-ends. One of two primers was radioactively labeled, preferably the *Eco*RI-primer. The primers were end-labeled using [γ-^{33}P]ATP and T4 polynucleotide kinase. The labeling reactions were performed in 50 µl 25 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 0.5 mM spermidine–3HCl using 500 ng oligonucleotide primer, 100 µCi [γ-^{33}P]ATP and 10 U T4 polynucleotide kinase. Twenty µl PCRs were performed containing 5 ng labeled *Eco*RI-primer (0.5 µl from the labeling reaction mixture), 30 ng *Mse*I-primer, 5 µl template-DNA, 0.4 U *Taq* polymerase, 10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of all four dNTPs.

The PCR conditions differed depending on the nature of the selective extensions of the AFLP primers used for amplification. AFLP reactions with primers having none or a single selective nucleotide were performed for 20 cycles with the following cycle profile: a 30 s DNA denaturation step at 94°C, a 1 min annealing step at 56°C, and a 1 min extension step at 72°C. AFLP reactions with primers having two or three selective nucleotides were performed for 36 cycles with the following cycle profile: a 30 s DNA denaturation step at 94°C, a 30 s annealing step (see below), and a 1 min extension step at 72°C. The annealing temperature in the first cycle was 65°C, was subsequently reduced each cyle by 0.7°C for the next 12 cycles, and was continued at 56°C for the remaining 23 cycles. All amplification reactions were performed in a PE-9600 thermocycler (Perkin Elmer Corp., Norwalk, CT, USA).

AFLP fingerprinting of complex genomes generally involved an amplification in two steps. The first step of this amplification procedure, named preamplification, was performed with two AFLP primers having a single selective nucleotide as described above, with the exception that 30 ng of both AFLP primers was used, and that these primers were not radioactively labeled. After this preamplification step, the reaction mixtures were diluted 10-fold with 10 mM Tris–HCl, 0.1 mM EDTA pH 8.0, and used

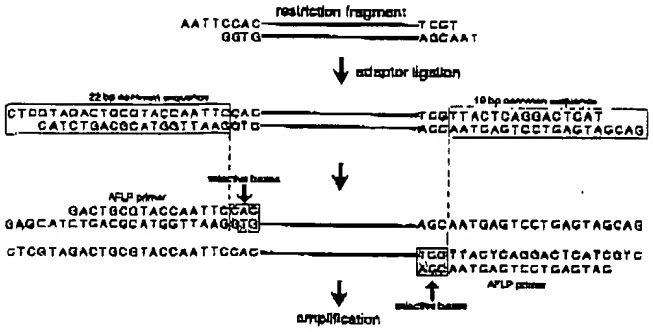


Figure 1. Schematic representation of the AFLP technique. Top: *EcoRI–MseI* restriction fragment with its 5′ protuding ends. Center: the same fragment after ligation of the *EcoRI* and *MseI* adapters. Bottom: both strands of the fragment with their corresponding AFLP primers. The 3′ end of the primers and their recognition sequence in the *EcoRI–MseI* fragment are highlighted.

as templates for the second amplification reaction. The second amplification reaction was performed as described above for AFLP reactions with primers having longer selective extensions.

Gel analysis

Following amplification reaction products were mixed with an equal volume (20 µl) of formamide dye (98% formamide. 10 mM EDTA pH 8.0. and bromo phenol blue and xylene cyanol as tracking dyes). The resulting mixtures were heated for 3 min at 90°C, and then quickly cooled on ice. Each sample (2 µl) was loaded on a 5% denaturing (sequencing) polyacrylamide gel (27). The gel matrix was prepared using 5% acrylamide, 0.25% methylene bisacryl, 7.5 M urea in 50 mM Tris/50 mM Boric acid/1 mM EDTA. To 100 ml of gel solution 500 µl of 10% APS and 100 µl TEMED was added and gels were cast using a SequiGen 38 × 50 cm gel apparatus (BioRad Laboratories Inc., Hercules, CA, USA). 100 mM Tris/100 mM Boric acid/2 mM EDTA was used as running buffer. Electrophoresis was performed at constant power, 110 W, for ~2 h. After electrophoresis, gels were fixed for 30 min in 10% acetic acid dried on the glass plates and exposed to Fuji phosphoimage screens for 16 h. Fingerprint patterns were visualized using a Fuji BAS-2000 phosphoimage analysis system (Fuji Photo Film Company Ltd, Japan).

RESULTS AND DISCUSSION

Principle of the method

The AFLP technique is based on the amplification of subsets of genomic restriction fragments using PCR. DNA is cut with restriction enzymes, and double-stranded (ds) adapters are ligated to the ends of the DNA-fragments to generate template DNA for amplification. The sequence of the adapters and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments (Fig. 1). Selective nucleotides are included at the 3′ ends of the PCR primers. which therefore can only prime DNA synthesis from a subset of the restriction sites. Only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified (Fig. 1).

The restriction fragments for amplification are generated by two restriction enzymes, a rare cutter and a frequent cutter. The AFLP

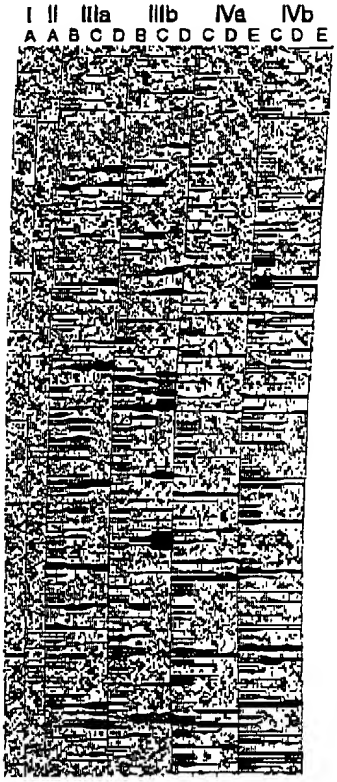


Figure 2. AFLP fingerprints of genomic DNAs of various complexities: λ DNA (panel I), AcNPV DNA (panel II), Acinetobacter DNA (panels IIIa and IIIb) and yeast DNA (panels IVa and IVb). Letters A, B, C, D and E refer to none, one, two, three and four selective bases in the AFLP primers respectively. The primer combinations used were from left to right: I. *EcoRI+0/MseI+0*, II. *EcoRI+0/MseI+0*, IIIa. *EcoRI+0/MseI+A*, *EcoRI+C/MseI+A*, *EcoRI+C/MseI+AT*. IIIb. *EcoRI+0/MseI+T*, *EcoRI+C/MseI+T*, *EcoRI+C/MseI+TA*. IVa. *EcoRI+C/MseI+G*, *EcoRI+C/MseI+GC*, *EcoRI+CA/MseI+GC*, IVb. *EcoRI+C/MseI+T*, *EcoRI+C/MseI+TA*. *EcoRI+CA/MseI+TA*. (+0 indicates no selective nucleotides, +A indicates selective nucleotide = A, etc). The molecular weight size range of the fingerprints is 45–500 nucleotides.

procedure results in predominant amplification of those restriction fragments, which have a rare cutter sequence on one end and a frequent cutter sequence on the other end (this will be explained below, see also Fig. 3). The rationale for using two restriction enzymes is the following. (i) The frequent cutter will generate small DNA fragments, which will amplify well and are in the optimal size range for separation on denaturing gels (sequence gels). (ii) The number of fragments to be amplified is reduced by using the rare cutter, since only the rare cutter/frequent cutter fragments are amplified. This limits the number of selective nucleotides needed for selective amplification. (iii) The use of two restriction enzymes makes it possible to label one strand of the ds PCR products, which prevents the occurence of 'doublets' on the gels due to unequal mobility of the two strands of the amplified fragments. (iv) Using two different restriction enzymes gives the greatest flexibility in 'tuning' the number of fragments to be amplified. (v) Large numbers of different fingerprints can be generated by the various combinations of a low number of primers.

AFLP fingerprinting of simple genomes

The AFLP techniquc makes use of ds adapters ligated to the ends of the restriction fragments to create target sites for primer

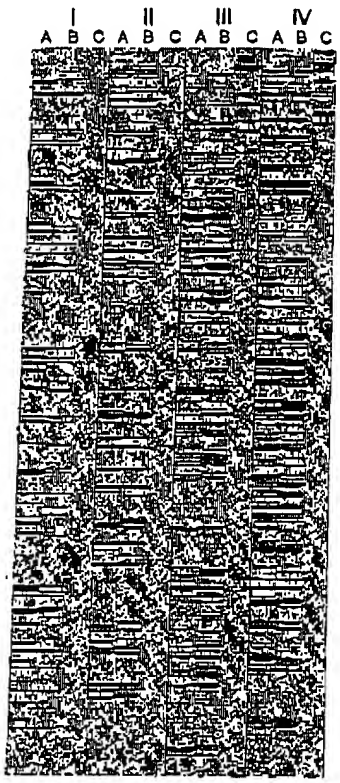


Figure 3. Illustration of the principle of preferential amplification of *Eco*RI–*Mse*I fragments. AFLP fingerprints are shown of yeast DNA using primer combinations with a single selective base for the *Eco*RI primers and two selective bases for the *Mse*I primer. Panels I, II, III and IV refer to the primer combinations *Eco*RI+C/*Mse*I+AC, *Eco*RI+C/*Mse*I+CA, *Eco*RI+T/*Mse*I+AG and *Eco*RI+T/*Mse*I+AT, respectively. Lanes A and B show standard AFLP fingerprints with either the *Eco*RI primers labeled (lanes A) or the *Mse*I primers labeled (lanes B). In lanes C the *Mse*I primers were labeled but the *Eco*RI primers were omitted from the AFLP reaction. The molecular weight size range of the fingerprints is from 80 to 500 nucleotides.

annealing in fragment amplification. A subset of the restriction fragments is specifically amplified by the use of selective nucleotides at the 3′ ends of the AFLP primers. This principle was demonstrated by fingerprinting of four different simple genomic DNAs, varying in genome size from 48.5 to 16 000 kb. The following genomic DNAs were selected and fingerprinted using the enzyme combination *Eco*RI/*Mse*I: phage λ DNA (48 451 bp) (29), AcNPV DNA (*Autographa californica* Nuclear Polyhedrosis Virus, 129 981 bp) (30), Acinetobacter DNA (estimated genome size 3000 kb) (31) and yeast DNA (estimated genome size 16 000 kb) (32) (Fig. 2).

For the DNAs of phage λ and AcNPV, the complete nucleotide sequence is known, and therefore all *Eco*RI/*Mse*I fragments could be exactly predicted. Indeed, all predicted fragments were detected. Also for Acinetobacter and yeast DNA, the number of fragments corresponded well with the genome sizes of these organisms. Adding selective nucleotides to the AFLP primers reduced the number of bands ~4-fold with each additional selective base. Furthermore, the addition of an extra selective nucleotide always resulted in a fingerprint, which was a subset of the original fingerprint. This indicates that the selective nucleotides are an accurate and efficient way to select a specific set of restriction fragments for amplification. The AFLP fingerprints showed that large numbers of restriction fragments were amplified simultaneously, and that in principle the number of bands detected is limited only by the power of the detection system, i.e. polyacrylamide gels. In general, the simultaneous amplification of DNA fragments using specific primer sets for each PCR fragment (multiplex PCR) appears to be rather troublesome (33–35). Our results suggest that multi-fragment amplification is efficient provided that all fragments use the same primer set for their amplification. This implies that the differences in amplification efficiency of DNA fragments in PCR (33–35), are mainly primer-associated and not fragment specific.

The ds adapters used for ligation to the restriction fragments are not phosphorylated, which causes only one strand to be ligated to the ends of the restriction fragments. Fragments were therefore not amplified if the template DNAs were denatured prior to PCR amplification (results not shown), except when *Taq* polymerase and dNTPs were present before denaturation. The filling-in of the 3′ recessed ends by the *Taq* polymerase following the dissociation of the non-ligated strands during the heating step seems a matter of only seconds or less. Alternatively, the *Taq* polymerase may immediately displace the non-ligated strands at low temperatures in the process of assembling the reaction mixtures.

These results demonstrated that: (i) the AFLP technique provides an efficient way to amplify large numbers of fragments simultaneously, (ii) the amplified fragments are restriction fragments, (iii) the number of fragments obtained increased as the genome size increased and corresponded well with what was theoretically expected, (iv) the selective nucleotides at the ends of the AFLP primers reduced the number of bands precisely as would be expected.

Restriction of the genomic DNA with *Eco*RI and *Mse*I will result in three classes of restriction fragments, *Mse*I–*Mse*I fragments, *Eco*RI–*Mse*I fragments and *Eco*RI–*Eco*RI fragments. The vast majority (>90%) are expected to be *Mse*I–*Mse*I fragments, the *Eco*RI–*Mse*I fragments will be about twice the number of *Eco*RI restriction sites and a small number of the fragments will be *Eco*RI–*Eco*RI fragments. In the previous experiments the *Eco*RI primer was labeled and, therefore, only the restriction fragments with an *Eco*RI site could be detected. To investigate the amplification of the *Mse*I–*Mse*I fragments a set of AFLP reactions on yeast DNA was performed with the *Mse*I primer labeled instead of the *Eco*RI primer (Fig. 3); this is expected to show the *Eco*RI–*Mse*I fragments as well as the *Mse*I–*Mse*I fragments.

Similar yeast fingerprints were obtained with either the *Eco*RI or *Mse*I primer labeled (Fig. 3, compare lanes A and B). However, most of the bands showed a significant shift in mobility, due to the fact that the other strand of the fragments was detected. It was surprising to find that almost no additional fragments, i.e. *Mse*I–*Mse*I fragments, were detected upon labeling of the *Mse*I primer instead of the *Eco*RI primer. Therefore, additional reactions were performed to which only the *Mse*I primer was added, which will theoretically only allow amplification of *Mse*I–*Mse*I fragments (Fig. 3, C lanes). Indeed, these reactions showed amplification products not observed in the presence of the *Eco*RI primers. These observations imply that amplification of the *Mse*I–*Mse*I fragments is inefficient in the presence of the *Eco*RI primer, i.e. that there is preferential amplification of *Eco*RI–*Mse*I fragments compared with the *Mse*I–*Mse*I fragments in the AFLP reaction. This may be explained in two ways. (i) The

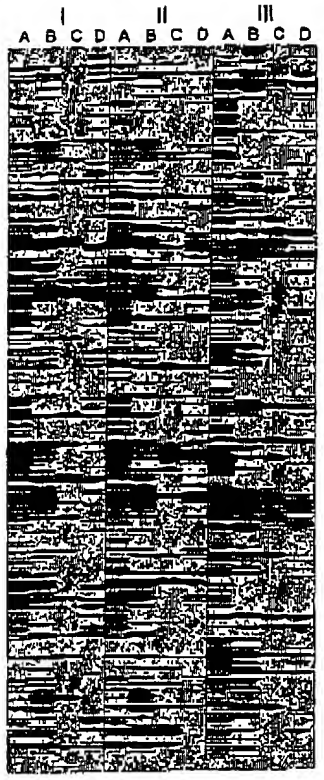


Figure 4. Illustration of the selectivity of AFLP primers. AFLP fingerprints are shown of yeast DNA using primer combinations with one selective base for the *Eco*RI primer and one (lanes A), two (lanes B), three (lanes C) and four (lanes D) selective nucleotides respectively for the *Mse*I primer. The primer combinations used are *Eco*RI+A/*Mse*I+CTCA (panel I), *Eco*RI+A/*Mse*I+CTGC (panel II) and *Eco*RI+T/*Mse*I+CTCA (panel III). The molecular weight size range of the fingerprints is from 40 to 370 nucleotides.

*Mse*I primer has a lower annealing temperature than the *Eco*RI primer, making amplification of *Mse*I–*Mse*I fragments less efficient compared with *Eco*RI–*Mse*I fragments under the conditions used. We have indeed observed stronger amplification of *Mse*I–*Mse*I fragments using longer or alternative *Mse*I primers and adapters. (ii) The *Mse*I–*Mse*I fragments have an inverted repeat at the ends, due to the fact that they are amplified by a single primer. Therefore, a stem–loop structure may be formed by base-pairing of the ends of the fragments, which will compete with primer annealing. This is also confirmed by the observation that only larger fragments were amplified in AFLP reactions when only the *Mse*I primer was added. Formation of a stem–loop structure will be more difficult in these larger fragments. We have indeed demonstrated that amplification of large fragments (>1 kb) with a single primer is quite efficient (results not shown).

Careful primer design is crucial for succesful PCR amplification. AFLP primers consist of three parts: the 5′ part corresponding to the adapter, the restriction site sequence and the 3′ selective nucleotides. Therefore, the design of AFLP primers is mainly determined by the design of the adapters, which are ligated to the restriction fragments. Various adapter designs were tested (results not shown), all with good results provided that the general rules for 'good PCR primer design' were followed (36,37). A different adapter design will demand different PCR conditions, and it is therefore important to select a specific design for the adapters, and to optimize the conditions for this design. An important feature of the AFLP primers is that all primers start with a 5′ guanine (G) residue. We have found that a 5′ G-residue in the unlabeled primer is crucial to prevent the phenomenon of double bands. The double bands appeared to result from incomplete addition of an extra nucleotide to the synthetised strands (results not shown). This terminal transferase activity of the *Taq* polymerase is quite strong, and has been frequently reported (38–40). We have also found that the 3′ nucleotide additions were influenced by the concentration of dNTPs, and that double bands occur at low concentrations of dNTPs regardless of the 5′-residue in the PCR primers (results not shown). We conclude from our results that this terminal transferase activity is most strong (almost 100% of the synthesized strands) when the 3′ nucleotide of the synthesized strand is a cytidine (C).

In the experiments described so far, AFLP primers were used with one or two selective 3′ nucleotides. This low number of selective nucleotides was shown to provide an effective way to select the desired number of fragments for amplification. Next, primers with longer 3′ extensions were tested to determine the maximum number of 3′ selective nucleotides which would retain high selectivity in AFLP reactions. For this purpose, fingerprints were generated of yeast DNA using primers with up to four selective nucleotides. A single *Eco*RI primer was selected with one selective nucleotide and combined with four different *Mse*I primers with one, two, three or four selective nucleotides, respectively. The *Mse*I selective extensions were choosen in a way that with each additional selective nucleotide a fingerprint would be generated that is the subset of the preceding fingerprints, e.g. extensions +C, +CT, +CTC, +CTCA. The appearance of bands that do not occur in the preceding fingerprints is an indication that fragments are amplified which do not correspond to the sequence of the selective bases, and consequently that selectivity is incomplete (Fig. 4).

The fingerprints shown in Figure 4 and the fingerprints from previous experiments demonstrated that primer selectivity is good for primers with one or two selective nucleotides. Selectivity is still acceptable with primers having three selective nucleotides, but it is lost with the addition of the fourth nucleotide. The loss of selectivity with primers having four selective bases is illustrated by the amplification of numerous bands not detected in the corresponding fingerprints with primers having three selective bases (compare lanes D with lanes C). This indicates the tolerance of mismatches in the amplification of the fragments using AFLP primers with four-base extensions. It is most likely that mismatches will be tolerated at the first selective base, because this nucleotide is positioned most distant from the 3′ end, and because the selectivity of three-base selective extensions was still adequate. Other researchers have also investigated the selectivity of the 3′ nucleotides of PCR primers (41,42). They have found that mismatches were tolerated at both the 3′ ultimate and penultimate nucleotides of PCR primers, which is in conflict with our findings. In other experiments, we have found that primer selectivity is relative and also depends on the number of fragments amplified in a single reaction, the PCR conditions and the primer design (results not shown). We feel that some level of mismatch amplification will always occur, but that the reaction conditions can prevent these mismatch bands to reach the

detection level. This, presumably, is the major difference with previously reported experiments (41,42).

AFLP fingerprinting of complex genomes

Initial experiments with AFLP fingerprinting of a number of plant and animal DNAs indicated that AFLP primers with at least three selective nucleotides at both the *Eco*RI and *Mse*I primer were required to generate useful band patterns. Because primers with three selective bases tolerate a low level of mismatch amplification, a two-step amplification strategy was developed for AFLP fingerprinting of complex DNAs. In the first step, named preamplification, the genomic DNAs were amplified with AFLP primers both having a single selective nucleotide. Next the PCR products of the preamplification reaction were diluted and used as template for the second AFLP reaction using primers both having three selective nucleotides. We have compared this amplification strategy with a direct amplification of complex genomic DNAs without the use of the preamplification step. The two-step amplification strategy resulted in two important differences compared with the direct AFLP amplification: (i) background 'smears' in the fingerprint patterns were reduced, and (ii) fingerprints with particular primer combinations lacked one or more bands compared with fingerprints generated without preamplification. This is best explained assuming that the direct amplification with AFLP primers having three selective nucleotides resulted in a low level of mismatch amplification products, which caused the background smears and gave discrete amplified fragments corresponding to repeated restriction fragments. An additional advantage of the two-step amplification strategy is that it provides a virtually unlimited amount of template DNA for AFLP reactions. Figure 5 shows a number of typical AFLP fingerprints obtained with the two-step amplification strategy using DNAs of three plant species, *Arabidopsis thaliana*, tomato (*Lycopersicon esculentum*) and maize (*Zea mays*), and human DNA. For Arabidopsis DNA primer combinations with a total of five selective nucleotides were used, because of the small genome of this plant species (145 Mb, 43). For tomato, maize and human DNA, six selective nucleotides were used, three selective bases for both the *Eco*RI and *Mse*I-primer.

DNA fingerprinting methods based on amplification of genomic DNA fragments by random primers have been found to be quite susceptible to the template DNA concentration (13,14). DNA quantities may vary considerably between individual samples isolated by standard DNA isolation procedures. Preferably, a DNA fingerprinting technique should be insensitive to variations in DNA template concentration. Therefore, the sensitivity of the AFLP technique for the template DNA concentrations was investigated.

AFLP fingerprints were performed using tomato DNA and the enzyme combination *Eco*RI/*Mse*I. Template DNA quantities were varied from 2.5 pg to 25 ng. The standard two-step amplification protocol was used, with the exception of an extended preamplification step for the 2.5 and 25 pg DNA template samples. In tomato 2.5 pg of template DNA corresponds to approximately four molecules of each DNA fragment at the start of the AFLP reaction. AFLP fingerprint patterns were very similar using template quantities that ranged 1000-fold, i.e. from 25 ng to 25 pg (Fig. 6). Fingerprints generated with only 2.5 pg of template DNA were similar to the other fingerprints, although bands varied significantly in intensity and some bands were absent.

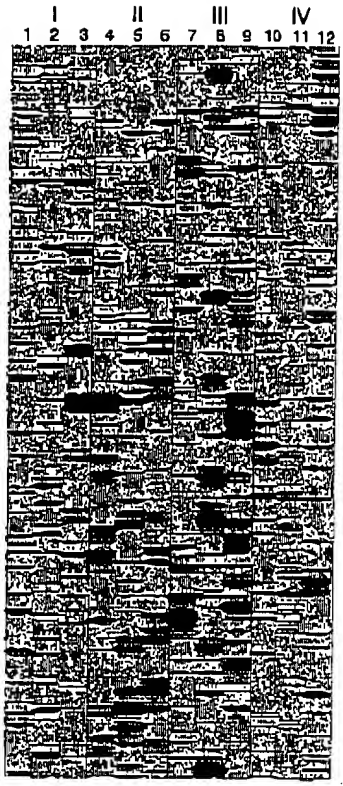


Figure 5. AFLP fingerprints of plant DNAs and human DNAs. Each panel shows three *Eco*RI–*Mse*I fingerprints using three different primer combinations. Human DNA fingerprints are displayed in panel IV, plant fingerprints are displayed in panels I (Arabidopsis), II (tomato) and III (maize), respectively. Primer combinations are from left to right: 1. *Eco*RI+CA/*Mse*I+CTT, 2. *Eco*RI+CA/*Mse*I+CAT, 3. *Eco*RI+CA/*Mse*I+CTC, 4. *Eco*RI+ACC/*Mse*I+CTT, 5. *Eco*RI+ACC/*Mse*I+CTC, 6. *Eco*RI+ACC/*Mse*I+CTA, 7. *Eco*RI+ACC/*Mse*I+CAT, 8. *Eco*RI+AGG/*Mse*I+CTT, 9. *Eco*RI+AGG/*Mse*I+CAA, 10. *Eco*RI+CAC/*Mse*I+CGA, 11. *Eco*RI+CAC/*Mse*I+CAA, 12. *Eco*RI+CAG/*Mse*I+CGA. The molecular weight size range of the fingerprints is from 45 to 500 nucleotides.

These results demonstrate that the AFLP procedure is insensitive to the template DNA concentration, although abberant fingerprints will be observed at very high template dilutions giving only a few template molecules at the start of the reaction. Most probably the individual restriction fragments are not randomly distributed at such low DNA concentrations explaining the observed differences in band intensities. This hypothesis is supported by our finding that comparison of a number of individual AFLP fingerprints obtained with only 2.5 pg of template DNA showed high variation in the intensity of the individual bands (results not shown).

A remarkable characteristic of the AFLP reaction is that generally the labeled primer is completely consumed (the unlabeled primer is in excess), and that therefore the amplification reaction stops when the labeled primer is exhausted. We have also found that further thermo cycling does not affect the band patterns once the labeled primer is consumed (results not shown). This characteristic is elegantly utilyzed in the AFLP protocol, which uses an excess of PCR cycles, which will result in fingerprints of equal intensity despite of variations in template concentration.

We have also noted that DNA fingerprints tend to be unreliable if the template concentration was below a certain absolute concentration (~1 pg), regardless of the complexity of the DNA

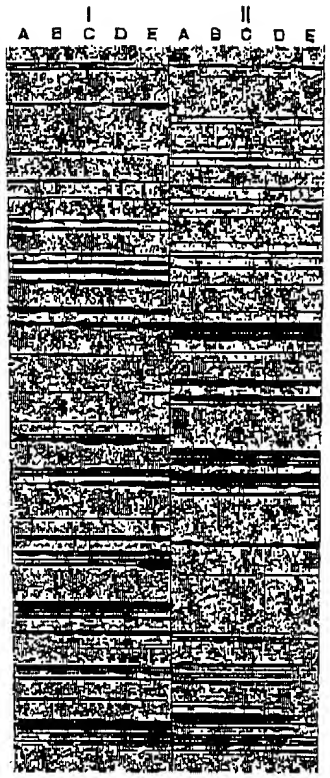


Figure 6. Illustration of the effect of template DNA concentration on AFLP fingerprinting. *Eco*RI–*Mse*I fingerprints of tomato DNA are shown using two primer combinations *Eco*RI+ACC/*Mse*I+CAT (panel I) and *Eco*RI+ACC/*Mse*I+CTT (panel II). Five different DNA concentrations were used as AFLP templates: 25 ng (lanes A), 2.5 ng (lanes B), 250 pg (lanes C), 25 pg (lanes D) and 2.5 pg (lanes E). The molecular weight size range of the fingerprints is 40–370 nucleotides.

(results not shown). In the latter case, bands were observed which were template independent and which were also present if no DNA was added.

The use of other restriction enzyme combinations for AFLP fingerprinting of complex genomes was also investigated (results not shown). These enzyme combinations included *Eco*RI, *Hin*dIII, *Pst*I, *Bgl*II, *Xba*I and *Sse8387*I (eight base cutter) in combination with either *Mse*I or *Taq*I. Fingerprints were generated on a variety of plant and animal DNAs. The use of *Taq*I as a four-base cutter in stead of *Mse*I resulted in an unequal distribution of the amplified fragments, which were mainly present in the upper part of the gel. Most eukaryotic DNAs are AT-rich, and as a result *Mse*I (recognition sequence TTAA) will generally produce much smaller restriction fragments compared with *Taq*I (recognition sequence TCGA). *Mse*I is therefore preferred for AFLP fingerprinting because it cuts very frequently in most eukaryotic genomes yielding fragments that are in the optimal size range for both PCR amplification and separation on denaturing polyacrylamide gels. Other rare cutter enzymes generally performed equal to *Eco*RI in AFLP fingerprinting, and the number of bands obtained reflected the cleavage frequency of the various restriction enzymes. However, *Eco*RI is preferred because it is a reliable (low cost) six-cutter enzyme, which limits problems associated with partial restriction in AFLP fingerprinting (see below).

Incomplete restriction of the DNA will cause problems in AFLP fingerprinting, because partial fragments will be generated, which will be detected by the AFLP procedure. When various DNA samples are compared with AFLP fingerprinting, incomplete restriction will result in the detection of differences in band patterns, which do not reflect true DNA polymorphisms, i.e. when one sample is partially restricted and the others are not. Incomplete restriction will only become apparent when different DNA samples of the same organism are compared. It is characterised by the presence of additional bands in the lanes, predominantly of higher molecular weight.

CONCLUSIONS

AFLP is a DNA fingerprinting technique that detects genomic restriction fragments and resembles in that respect the RFLP (restriction fragment length polymorphism) technique, with the major difference that PCR amplification instead of Southern hybridisation is used for detection of restriction fragments. The resemblance with the RFLP technique was the basis to chose the name AFLP. The name AFLP, however, should not be used as an acronym, because the technique will display presence or absence of restriction fragments rather than length differences.

In our initial AFLP protocols an additional purification step was incorporated in the template preparation (28). Biotinylated *Eco*RI-adapters were used for ligation, and subsequently the biotinylated DNA fragments (*Eco*RI–*Mse*I fragments and *Eco*RI–*Eco*RI fragments) were substracted from the ligation mixture using streptavidin beads (28,44). This step reduced the complexity of the DNA template by removing all *Mse*I–*Mse*I fragments, which proved to be important for generating high quality fingerprints of complex DNAs. The protocol described in this paper omits this purification step, because amplification conditions are used which result in preferential amplification of *Eco*RI–*Mse*I fragments with respect to the *Mse*I–*Mse*I fragments (Fig. 3). This is the result of careful adapter and primer design and inherent characteristics of the AFLP amplification reaction.

The AFLP technique will generate fingerprints of any DNA regardless of the origin or complexity, and in this paper we have presented AFLP fingerprints of DNAs differing in genome size as much as 100 000-fold. We have described that the number of amplified fragments may be controlled by the cleavage frequency of the rare cutter enzyme and the number of selective bases. In addition the number of amplified bands may be controlled by the nature of the selective bases; selective extensions with rare di- or trinucleotides will result in the reduction of amplified fragments. In general, there is an almost linear correlation between numbers of amplified fragments and genome size. This linear correlation is lost in the complex genomes of higher plants, which contain high numbers of repeated sequences and, hence, multicopy restriction fragments. Fingerprints of these complex DNAs consist predominantly of unique AFLP fragments, but are characterised by the presence of small numbers of more intense repeated fragments.

In complex genomes the number of restriction fragments that may be detected by the AFLP technique is virtually unlimited. A single enzyme combination (a combination of a specific six-base and four-base restriction enzyme) will already permit the amplification of 100 000s of unique AFLP fragments, of which generally 50–100 are selected for each AFLP reaction. Most AFLP fragments correspond to unique positions on the genome, and, hence, can be exploited as landmarks in genetic and physical

maps, each fragment being characterized by its size and its primers required for amplification. In addition, the AFLP technique permits detection of restriction fragments in any background or complexity, including pooled DNA samples and cloned (and pooled) DNA segments. Therefore, the AFLP technique is not simply a fingerprinting technique, it is an enabling technology in genome research, because it can bridge the gap between genetic and physical maps. First, the AFLP technique is a very effective tool to reveal restriction fragment polymorphisms. These fragment polymorphisms, i.e. AFLP markers, can be used to construct high density genetic maps of genomes or genome segments. In most organisms AFLP will prove to be the most effective way to construct genetic DNA marker maps compared to other existing marker technologies. Secondly, the AFLP markers can be used to detect corresponding genomic clones, e.g. yeast artificial chromosomes (YACs). This is most effectively achieved by working with libraries, which are pooled to allow for rapid PCR screening and subsequent clone identification. An AFLP marker will detect a single corresponding YAC clone in pools of as much as 100 YAC clones (M. Zabeau, M. Kuiper and P. Vos., manuscript in preparation). Finally, the AFLP technique may be used for fingerprinting of cloned DNA segments like cosmids, P1 clones, bacterial artificial chromosomes (BACs) or YACs (results not shown). By simply using no or few selective nucleotides, restriction fragment fingerprints will be produced, which subsequently can be used to line up individual clones and make contigs.

ACKNOWLEDGEMENTS

We would like to thank Richard Michelmore, Hanneke Witsenboer and Francesco Salamini for critically reading the manuscript. This work was financed by Keygene N.V. and six Dutch breeding companies: Royal Sluis, Cebeco Zaden, ENZA Zaden, De Ruiter Seeds, Rijkzwaan and Ropta ZPC. The work was subsidized by the Dutch ministery of economic affairs through a PBTS grant.

Note: The AFLP technique is covered by patents and patent applications owned by Keygene N.V. Information concerning licenses to practice the AFLP process for commercial purposes can be obtained from Keygene N.V. Research kits for AFLP fingerprinting of plant genomic DNA are available from Life Technologies (Gathersburg, MD, USA) and Perkin Elmer (Applied Biosystems Division, Foster City, CA, USA).

REFERENCES

1 Jeffreys, A.J., Wilson, V. and Thein, S.L. (1985) *Nature*, **314**, 67–73.
2 Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., Culver, M., Martin, C., Fujimoto, E., Hoff, M., Kumlin, E. and White, R. (1987) *Science*, **235**, 1616–1622.
3 Jeffreys, A.J., Melcod, A., Tamaki, K., Neil, D.L. and Monckton, D.G. (1991) *Nature*, **354**, 204–209.
4 Tautz, D. (1989) *Nucleic Acids Res.*, **17**, 6463–6472.
5 Weber, J.L. and May, P.E. (1989) *Am. J. Hum. Genet.*, **44**, 388–396.
6 Edwards, A., Civitello, A., Hammond, H.A. and Caskey, C.T. (1991) *Am. J. Hum. Genet.*, **49**, 746–756.
7 Beyermann, B., Nurnberg, P., Weihe, A., Meixner, M., Epplen, J.T. and Börner, T. (1992) *Theor. Appl. Genet.*, **83**, 691–694.
8 Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990) *Nucleic Acids Res.*, **18**, 6531–6535.
9 Cactano-Anolles, G., Brassam, B.J. and Gresshoff, P.M. (1991) *Bio/Technology*, **9**, 553–557.
10 Welsh, J. and McClelland, M. (1990) *Nucleic Acids Res.*, **18**, 7213–7218.
11 Welsh, J. and McClelland, M. (1991) *Nucleic Acids Res.*, **19**, 861–866.
12 Riedy, M.F., Hamilton, W.J.III. and Aquadro, C.F. (1992) *Nucleic Acids Res.*, **20**, 918.
13 Ellsworth, D.L., Rittenhouse, K.D. and Honeycutt, R.L. (1993) *BioTechniques*, **14**, 214–217.
14 Muralidharan, K. and Wakeland, E.K. (1993) *BioTechniques*, **14**, 362–364.
15 Micheli, M.R., Bova, R., Pascale, E. and D'Ambrosio, E. (1994) *Nucleic Acids Res.*, **22**, 1921–1922.
16 Cactano-Anolles, G., Brassam, B.J. and Gresshoff, P.M. (1992) *Mol. Gen. Genet.*, **235**, 157–165.
17 Botstein, D., White, R.L., Skolnick, M.H. and Davies, R.W. (1980) *Am. J. Hum. Genet.*, **32**, 314–331.
18 Tanksley, S.D., Young, N.D., Paterson, A.H. and Bonierbale, M.W. (1989) *Bio/Technology*, **7**, 257–264.
19 Mullis, K.B. and Faloona, F.A. (1987) *Methods Enzymol.*, **155**, 335–350.
20 Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Ehrlich, H.A. (1988) *Science*, **239**, 487–491.
21 Ehrlich, H.A., Gelfland, D.H. and Sninsky, J.J. (1991) *Science*, **252**, 1643–1650.
22 Vlak, J.M., Klinkenberg, F.A., Zaal, K.J.M., Usmany, M., Klinge-Roode, E.C., Geervliet, J.B.F., Roosien, J. and Van Lent, J.W.M. (1988) *J. Gen. Virol.*, **69**, 756–776.
23 Pitcher, D.G., Saunders, N.A. and Owen, R.J. (1989) *Lett. Appl. Microbiol.*, **8**, 151–156.
24 Green, E.D. and Olson, M.V. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 1213–1217.
25 Stewart, C.J., Jr and Via, L.E. (1993) *BioTechniques*, **14**, 748–750.
26 Miller, S.A., Dykes, D.D. and Polesky, H.F. (1988) *Nucleic Acids Res.*, **16**, 1215.
27 Maxam, A.M. and Gilbert, W. (1980) *Methods Enzymol.*, **65**, 499–560.
28 Zabeau, M. and Vos, P. (1993) *European Patent Application*, publication no: EP 0534858.
29 Sanger, F., Coulson, A.R., Hong, G.F., Hill, D.F. and Petersen, G.B. (1982) *J. Mol. Biol.*, **162**, 729–773.
30 Ayres, M.D., Howard, S.C., Kuzio, J., Lopez-Ferber, M. and Possee, R.D. (1994) *Virology*, **202**, 585–605.
31 Bouvet, P.J.M. and Grimont, P.A.D. (1986) *Int. J. Syst. Bacteriol.*, **36**, 228–240.
32 Curie, G.F. and Olson, M.V. (1984) *Nucleic Acids Res.*, **12**, 5647–5664.
33 Chamberlain, J.S., Gibs, R.A., Ranier, J.E., Nguyen, P.N. and Caskey, C.T. (1988) *Nucleic Acids Res.*, **16**, 11 141–11 156.
34 Rossiter, B.J.F., Fuscoe, J.C., Muzny, D.M., Fox, M. and Caskey, C.T. (1991) *Genomics*, **9**, 247–256.
35 Ferrie, R.M., Schwarz, M.J., Robertson, N.H., Vaudin, S., Super, M., Malone, G. and Little, S. (1992) *Am. J. Hum. Genet.*, **51**, 251–262.
36 Dieffenbach, C.W., Lowe, T.M.J. and Dveksler, G.S. (1993) *PCR Methods Applic.*, **3**, S30–S37.
37 Breslauer, K.J., Ronald, F., Blocker, H. and Marky, L.A. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 3746–3750.
38 Lawyer, F.C., Stoffel, S., Saiki, R.K., Myambo, K., Drummond, R. and Gelfand, D.H. (1989) *J. Biol. Chem.*, **264**, 6247–6437.
39 Clark, J.M. (1988) *Nucleic Acids Res.*, **16**, 9677–9686.
40 Hu, G. (1993) *DNA Cell Biol.*, **12**, 763–770.
41 Kwok, S., Kellogg, D.E., McKinney, N., Spasic, D., Goda, L., Levenson, C. and Sninsky, J.J. (1990) *Nucleic Acids Res.*, **18**, 999–1005.
42 Newton, C.R., Graham, A., Heptinstall, L.E., Powell, S.J., Summers, C., Kalsheker, N., Smith, J.C. and Markham, A.F. (1989) *Nucleic Acids Res.*, **17**, 2503–2516.
43 Arumuganathan, K. and Earle, E.D. (1991) *Plant Mol. Biol. Reporter*, **9**, 208–218.
44 Uhlen, M. (1989) *Nature*, **340**, 733–744.

TABLE 7.1 Guitarrero *Phaseolus* Varieties

	Median seed dimensions (cm)				
	Length	Width	Thickness	Number of measurable seeds	Morphology and distribution
P. vulgaris					
Pv 1	1.09	.74	.55	30	Dark plum, self-colored, spherical purple pod. Similar to Pichasca Pv 3.
Pv 4	1.00	.67	.43	9.5	Dark red-brown, self-colored kidney, ends round. Same as Pichasca Pv 1, similar to late Ayacucho.
Pv 5	1.29	.83	.56	19	Similar to Pv 4.
Pv 6	1.1	.79	.69	4	Yellow, spherical. New to archaeology.
P. lunatus					
Pl 2	1.65	1.19	.42	8	Tan, mottled eye. Similar to but smaller than *P. lunatus* from Early Nasca 200 A.D. Ica Valley and later.
Pl 3	1.57	1.22	.43	9	Black. New to archaeology.

in seed size. However, there is as yet no archaeological documentation of this event or trend. No wild *P. vulgaris* seeds have been reported in any archaeological site, and there is no trend toward size increase observable in any single bean race that occurs over a long time span in any reported site. The eastern Andean slopes where wild *P. vulgaris* (*P. aborigineus*) ranges from 1000 to 2800 m above sea level has had little paleoethnobotanical investigation and

TABLE 7.2 Distribution of Beans by Stratigraphic Complex

	Number of seeds of *P. vulgaris* varietal types				Number of seeds of *P. lunatus* varietal types	
Complex	Pv 1	Pv 4	Pv 5	Pv 6	Pl 2	Pl 3
IV	21 ± 1	2.5	17	4	2	7
		Numerous Pv pods			4–5 Pl pods	
III	2 ± 1	–	1	–	3	–
II?	3	–	1	–	2	2
					About 100 Pl pods	
II	4	1	–	–	1	–
I	–	–	–	–	–	–

Papers presented in SEMINAR on

(10)

(17)

(17)

Potentials of field beans and other food legumes in Latin America

SERIES SEMINARS No. 2E

Cali, Colombia, February 26 - March 1, 1973

Centro Internacional de Agricultura Tropical

Hno. Rigo por todo el amor de sus "potentials"

SERIES SEMINARS No. 2E

Potentials of field beans and other food legumes in Latin America

February 26- March 1, 1973

Centro Internacional de Agricultura Tropical
Cali, Colombia

I. Commentary upon:

PLANT INTRODUCTION AND GERMPLASM OF *PHASEOLUS VULGARIS* AND OTHER FOOD LEGUMES

Efraim Hernandez X.

INTRODUCTION

The quality of a germplasm bank depends basically on a well meditated and executed ethnobotanical exploration conducted during successive periods in accordance with concomitant phytogeographical and genetic studies of the materials collected. With regard to this point, Hernández X. (1970) has suggested the following guidelines:

(1). There are antecedents which should be reviewed (botanical, phytogeographical, ethnic, etc.) as a basis for the planning of the exploration.

(2). The environment, both physical and biotic, determines the geographic distribution of the crops and the genetic diversity.

(3). Man is and has been the most important factor in determining the genetic diversity of the cultigens.

(4). Each species or variety has distinctive morphological and adaptive characteristics.

(5). The knowledge accumulated throughout the millenia by the agricultural cultures takes time to register and understand.

(6). The ethnobotanical exploration is a dialectic process.

Keeping these points in mind, it should be noted that the greater part of the bean collections to date have been made by explorers interested in other crops, devoted to other scientific interests and in rare instances have they had a specific knowledge of beans. There are several studies of Phaseolus vulgaris (Miranda C., 1966), of P. lunatus (Mackie, 1943) and of P. acutifolius (Freeman, 1918) which are helpful in obtaining initial information on the morphological variation of the seed. Burkart (1952) shows that this structure is of great use to differentiate the existing variation through its morphological characteristics.

We insist on the seed, since this is easy to collect, it is the part that is usually available and it can be handled with facility for the desired purpose. The procedure followed to date for collecting results in partial data and ignorance of the phenomena because the materials collected are of the commercial types available in the markets. This situation can be improved only through a special effort to visit the regional markets and insisting upon collecting material directly from the farmers.

Obtaining knowledge of the multiple ecological and social niches of a given area and collections in those that might be of interest require time that is not often available to the plant explorer. For this reason, some of the most interesting series of collections have been made by ethnobotanists or anthropologists who have lived for some time in ethnic areas under study.

If plant exploration is coordinated with an active center of genetic research, a feedback system may be established to keep the explorer informed as to when he might be in an area especially interesting due to the genetic variation found and the morphological characteristics which might serve as indicators. This type of arrangement enabled Dr. Howard S. Gentry quickly to locate the center of diversity of the type of phytopathological resistance desired during his bean collecting trips to Mexico.

THE *PHASEOLUS VULGARIS* GERMPLASM BANKS IN MEXICO

Table 1 summarizes the available information on the germplasm banks in Mexico, complementing the information presented by Dr. Glibas Vieira. Due to my personal participation in establishing these banks, I am in a position to make the following observations:

(1). The greater part of the collections represent contributions by persons indirectly concerned with beans and these collections have not been increased in number, as a result of the study of the material, with the exception of the wild species which are of specific interest to Ing. Salvador Miranda Colín.

(2). These banks have never been under the care of personnel trained specifically for this purpose.

(3). The presence of these banks has not given origin to specific research programs dealing with the biology and phytogeography of the materials, to the increase in the number of accessions, etc.

(4). Although the collections have been reproduced several times, no uniform system of registering the data has been established, nor a system of rapid retrieval of information.

(5). Several studies have been made indicating various degrees of outcrossing in beans, but this knowledge has not been taken into account during the process of increasing the material to regain viability and maintain the genetic-variability.

Ethnobotanic exploration of beans has not been dynamic because the biosystematic studies which might put order in the existing genetic variation, indicate the morphological expression of this variation and show the geographic distribution of said variation, have just been initiated. This genetic, morphological and phytogeographical information is essential in planning future explorations.

Table 1. Phaseolus collections, classified by place of origin, and kept at the Instituto Nacional de Investigaciones Agrícolas, Secretaría de Agricultura y Ganadería (INIA), y del Colegio de Postgraduados, Escuela Nacional de Agricultura (CP), in Chapingo, Mexico, up to 1972.

Mexico	INIA	C. P.	Other countries	C. P.
Aguascalientes	180	26	Africa	24
Baja California	12	3	Argentina	9
Campeche	21	2	Brazil	14
Coahuila	17	42	Canada	2
Colima	13	8	Colombia	53
Chiapas	643	177	Costa Rica	12
Chihuahua	29	35	Cuba	17
Durango	33	53	Chile	3
Guanajuato	127	155	Ecuador	42
Guerrero	166	120	U. S. A.	48
Hidalgo	74	60	Guatemala	126
Jalisco	159	160	Honduras	16
Mexico	220	176	Japan	3
Michoacan	197	125	Peru	80
Morelos	65	150	Subtotal	449
Nayarit	41	25		
Nuevo Leon		1		
Oaxaca	265	115		
Puebla	765	414		
Queretaro	50	46		
Quintana Roo				
San Luis Potosí	25	26	INIA	3,765
Sinaloa	46	29	C.P. Mexico	2,274
Sonora	77	10	C. P. other countries	449
Tabasco	4			
Tamaulipas	13	10	Total	6,488
Tlaxcala	140	112		
Veracruz	230	112		
Yucatan	45	2		
Zacatecas	108	80		
Subtotales	3,765	2,274		

The Phaseolus germplasm bank of INIA has received important contributions from: Efraim Hernández X.; Salvador Miranda C.; Howard S. Gentry; George Freytag; Lawrence Kaplan and Alfonso Crispin.

The Phaseolus germplasm bank of C.P., ENA, has received important contributions from: Salvador Miranda and Efraim Hernández X. This bank has a large collection of wild species.

(Source: Register of Germplasm Banks).

For this reason, there is no basis for future explorations except for collecting in areas not represented in the material in the banks.

BIOSYSTEMATIC CONTRIBUTIONS ON BEANS

One of the most interesting biosystematic studies made to date (Hernandez X., Miranda C. and Prywer, 1959) indicates constant introgression between Phaseolus vulgaris and P. coccineus in transition areas between the hot humid and the temperate humid zones of Mexico and Guatemala. The importance of this information might be appreciated from the futile attempts made by researchers in Europe and the U. S. A. to incorporate resistance to insects and diseases from P. coccineus (perhaps the least domesticated of the bean species) to the common bean. We have evidence also that a natural cross between P. vulgaris and P. lunatus has occurred in the Jalisco-Michoacan regions of Mexico.

In our initial studies tending to define the morphological characters of the plant suitable for the differentiation of the varieties, we have found evidence of frequent introgression of a wild bean complex and the "upland black" bean complex, a phenomenon which does not appear in the collections of the "lowland black" bean complex.

EROSION OF GENETIC RESOURCES OF BEANS IN MESOAMERICA

The erosion of the genetic resources of primitive cultivars in Mesoamerica has caused concern especially on the part of international organizations which have before them the experience from areas where modern agricultural technology has eliminated the primitive varieties. The conclusions presented in a recent report to FAO (Hernández X., 1972, unpublished) based primarily on maize in Latin America, probably will give rise to certain doubts on the matter. Taking as a base the recent collections of maize in certain critical areas, it is concluded that after a period of 25 years, the same varieties persist and that new recombinations have appeared. These are being conserved in accordance with the new necessities of the ethnic groups arising from an increase in their agricultural areas and to changes in the edaphic conditions of their lands.

EROSION OF GENETIC RESOURCES

It seems that erosion of the genetic resources is a function of:

1. Cultural erosion. With greater degree of disorganization of the agricultural cultures, there is a greater degree of erosion of the genetic resources of the primitive cultivars. This means that the indigenous cultures are the ones that have conserved and given rise to the basic genetic diversity through their prolonged periods of existence. These cultures have conserved the greater part of the variants, eliminating only those that have been replaced with advantage by new types in the multiple ecological, economic and cultural niches of the ethnic group in question. These cultures, one should remember, are at the center of many of the attempts of change by the diverse Indian programs in Latin America.

2. Introduction and acceptance of improved varieties. This occurs especially in commercial agricultural systems. For the subsistence farmer, this process has a very reduced effect. This process might affect the selection under domestication or the quality of seed sown in accordance with the degree of commercialization of the regional agriculture. In Mexico, the establishment of the CONASUPO, an official institution involved in the maintenance of a minimum price for certain crops, among them beans, and in regulating prices of certain basic food products, has favored a preferential price for a uniform product and the bean types known as "canario," "bayo grande," "garrapata," "cacahuete," "flor de mayo" and "mantequilla or garbancillo." This commercial preference probably has displaced the multiple varieties produced previously in certain areas.

4. Degree of natural crosses. Beans had been considered an autogamous plant. Recent studies have demonstrated that natural outcrossing varies in accordance with ecological conditions and that a high enough percentage might occur to consider the species, through time, as an allogamous plant in the regions of its cultivation in Mesoamerica. Under subsistence agriculture, one finds the general practice of associating beans and maize and the use of a mixture ("revoltura") of seed as a possible answer to extremely fluctuating ecological conditions. Table 2 shows the components of a "revoltura" collected in the bean growing region of northeastern Puebla, Mexico.

Table 2. Composition of a mixture (composite sample) of bean seeds planted in the northeast region of the State of Puebla, Mexico, under uncertain climatic conditions, low soil fertility and in association with maize. Notice that two species of Phaseolus are involved.

Diameter in mm	Black	Black (white)	Red	Red (variegated)	Brown	Brown (variegated)	Gray Gray (variegated)	Yellow	Yellow (variegated)	Olive	Olive (variegated)	White White (black)	No. of seeds	Species
.5 to .55	21	8	3	89	26	1	2	19		3	3	4	179	Phas. vulgaris
.6 to .65	24	18	6		22	38	2/7	53		33		31 3	237	" "
.7	4	5	3		8	9	3/7	49				15	103	" "
.75	4	5		3			5/3	46				5	71	" "
.8	1				1		2	39				1	44	" "
.85	2					3	1	8					14	" "
.9	1						1	7					9	" "
.95					1		2	2/2					7	P. coccineus (3,4)
1.0	1				6			1					8	" "
1.1					1			2					3	" "
1.2						3							3	" "
Totals	58	36	12	92	65	54	12/22	225	3	36	3	56 3	678	

5. Opening of new agricultural areas. The increase of the human populations in almost all of the Latin American countries and the various attempts at agrarian reform have stimulated the opening for agricultural purposes of new areas, especially in the hot humid regions. This process has created a demand for new varieties, a demand which in the case of maize has been met by existing indigenous varieties and by the formation of new varieties by the indigenous cultures. As a result, this process has tended to increase the genetic variability.

6. Changes in the long-established agricultural areas. In spite of the programs of agricultural extension and the increase in agricultural inputs, large areas still face the problem of agricultural production under limiting ecological conditions and a low level of use of agricultural inputs. This has resulted in land erosion and a reduction of soil fertility after prolonged periods of agricultural utilization. As a consequence, one might forecast the selection by the farmers of varieties with capacity to insure the production of a minimum crop. This phenomenon will tend to increase the genetic variability.

STIMULUS FOR THE ETHNOBOTANICAL EXPLORER

One of the most important stimuli in science is the recognition of the researcher for his work. In the case of the botanical collectors, their names remain as essential information of the collection and in certain cases the generic and specific scientific names of the plants have had their origin on the collector's name. In contrast, we have noticed that the name of the ethnobotanist rapidly falls by the wayside in all of the schemes of management of the material of useful plants. For instance the collections of the U. S. Department of Agriculture are handled by means of the initial P. I.; the CIMMYT bank handles its material through abbreviation which indicate the political state of origin, for example, Ags. 7, Mich. 21, Tamps. 38; a similar procedure is followed by the banks of the National Institute of Agricultural Research (INIA) and of the Graduate College, E.N.A., both at Chapingo, Mexico. May I suggest a modification in the present systems by the addition, after the established abbreviations, of the collector's or collectors' surname in parentheses, for example, Mor. 592 (Miranda C.); P. I. 28970 (Gentry).

Voysest O. 1983. Variedades de frijol en America Latina y su origen. Centro Internacional de Agricultura Tropica Colombia. 87p.

(11) (8)

Tercero; proviene del cruce 'Jamapa' x 'P.I. 310814' obtenido en el CIAT y distribuido originalmente a Honduras con la siguiente genealogía: FF11-10-1-CM-C-M-CM(4-B)-CM.

El Cuadro 17 es una lista de las variedades de frijol sembradas en Honduras.

Jamaica

Las variedades de frijol que se cultivan en Jamaica se describen en el Cuadro 18.

Cuadro 18. **Variedades de frijol cultivadas en Jamaica.**

Variedades	Clase de grano		Origen
	Color	Tamaño	
Miss Kelly	rosado moteado	mediano	
Round Red	rojo	pequeño	
Charlevoix	rojo	mediano	Introducción de E.U.
Portland Red	rojo oscuro	pequeño	Introducción de E.U.
Long Red	rojo	mediano	
Tom Red	rojo	pequeño	
Cockstone	rosado moteado	grande	
ICA Duva	rojo	grande	Introducción de Colombia
ICA Guali	rojo moteado	grande	Introducción de Colombia
California Light Red Kidney	rosado claro	grande	Introducción de E.U.
California Dark Red Kidney	rosado oscuro	grande	Introducción de E.U.

México

La incontable riqueza genética del frijol mexicano dificulta la clasificación de los tipos cultivados en ese país; sin embargo, podría decirse que en México se cultivan comercialmente los siguientes frijoles:

- Canarios
- Azufrados — Azufrado; Azufrado peruano
- Bayos — Bayo Gordo (grano grande); Bayo Rata (grano grande); Garbancillo (grano mediano o pequeño)

•	Negros	Opacos (grano pequeño) Brillantes (grano mediano)
•	Pintos	Flor de Mayo Pinto Americano Ojo de Cara Cacahuate
•	Otros	Blancos (grano pequeño y mediano) Rojo (grano mediano o grande)

Esta variabilidad genética ha suscitado en México una intensa labor de selección en los materiales comerciales de color diferente al negro; además, se han obtenido variedades por hibridación.

La investigación en frijol se remonta en México a 1936 cuando se hicieron las primeras colecciones de material mexicano. En 1949, en Tlanepantla, México, se lograron los primeros cruces de frijol canario y bayo cuando se buscaban plantas arbustivas y de tipo III que demostraran resistencia a la roya, la antracnosis y la bacteriosis. En 1949 se entregaron las variedades 'Rocamex 1', 'Rocamex 2' y 'Rocamex 3' seleccionadas por Wellhausen y otros; posteriormente, estas variedades serían conocidas como 'Amarillo 154', 'Negro 150' y 'Bayo 158', respectivamente.

En 1955 se distribuyó semilla de la variedad 'Canario 101', un resultado de selecciones individuales de una colección reunida en Tacámbaro, Michoacán. Junto con esta variedad aparecieron 'Bayo 158', 'Bayo 160', 'Bayo 161', 'Negro 150', 'Negro 151' y 'Pinto 162', producto de selecciones individuales hechas en 1953. Las primeras variedades obtenidas por cruzamiento, 'Canocel', 'Bayomex', y 'Negro Mecentral', fueron lanzadas en 1959.

En 1958, en el Campo Experimental de Cotaxtla, Veracruz, se dieron a conocer tres nuevas variedades de frijol negro; 'Antigua', una selección de Guatemala, 'Actopán' y 'Jamapa'; esta última ha sido una de las más famosas variedades de frijol por su gran estabilidad, que ha mantenido durante más de 20 años. Fue desarrollada por F. Cárdenas partiendo de una selección de 15 líneas en la colección 'Veracruz 86', en Paso de Ovejas, Veracruz, en 1955.

A principios de la década del 70, el entonces Centro de Investigaciones Agrícolas de Sinaloa (hoy CIAPAN) empezó a distribuir nuevas variedades de frijol desarrolladas en ese centro por un equipo dirigido por Héctor López. Algunas fueron obtenidas por selección individual como 'Azufrado 33', y otras por hibridación como 'Canario 78' (Ahome), 'Azufrado 100' (Cahita 100), 'Culiacán 200', 'Azufrado Pimono 78' (Mayocoba), 'Canario 72' (CIAS 72) y 'Toche 400'.

En el centro-norte del país se han producido las variedades 'Pinto Nacional 73' (Pinamerpa), 'Bayo Calera', 'Delicias 71', 'Bayo Baranda', 'Bayo Durango', 'Pinto Fresnillo', y 'Ojo de Cabra 73' (Ciechi), principalmente. En el estado de Tamaulipas se obtuvieron las variedades 'Agramejo', 'Azabache', 'Mulato' y 'Ciateño'.

Se han citado los nombres de tres personas solamente en esta revisión de variedades para ubicar tres etapas cumplidas en el mejoramiento genético del frijol en México: la que corresponde a variedades mejoradas, hasta 1959; la aparición de 'Jamapa', una de las variedades de mayor difusión mundial; y una tercera, cubierta por el trabajo de Héctor López, prematuramente fallecido en 1978, quien fue protagonista de una era fructífera en el desarrollo de variedades en el estado de Sinaloa.

Dado el gran número de variedades producidas en México y la forma como está organizado su programa de frijol —coordinado por disciplinas a nivel nacional— es difícil acreditar a los fitomejoradores por la creación de variedades sin correr el riesgo de cometer omisiones graves. Cerca de 60 profesionales del Programa de Frijol del Instituto Nacional de Investigaciones Agrícolas (INIA) adelantan trabajos de investigación en 29 campos experimentales. El trabajo de mejoramiento genético se ha distribuido en cinco áreas ecológicas bien definidas; en cada una de ellas solamente un campo desarrolla el programa de mejoramiento genético, y es responsable de generar los materiales que serán distribuidos a campos distintos dentro de su área, donde son probados por equipos multidisciplinarios.

En el Cuadro 19 se presentan las principales variedades de frijol que se cultivan en México.

Nicaragua

Las variedades de frijol sembradas en Nicaragua hasta 1953 fueron todas criollas; de ese año en adelante se organizó el Programa de Mejoramiento que procedió a colectar material nacional y a introducir frijoles de México y Colombia.

En el sexenio 1956-1962 se produjeron 750 toneladas de frijol de la variedad 'Rico' —introducida de Costa Rica en 1952— que después de su evaluación fue seleccionada para sembrarla en gran escala. Del incansable esfuerzo de introducción y evaluación surgieron notables variedades como 'Jamapa' y 'Porrillo No. 1', cuya productividad sobrepasa las 2 t/ha de grano.

En 1965 se inicia la evaluación de la variedad 'Veranic-2', producto de una selección masal practicada en la variedad 'Jamapa'; esta última ha demostrado gran variabilidad genética ofreciendo muchas posibilidades de selección.

English translation of (8)

Mexico

The countless genetic richness of the Mexian beans makes difficult the classification of [all] cultivated types of this country. However one could say that the following bean varieties are grown commercially in Mexico:

* Canarios	
* Azufrados:	Azufrado
	Azufrado peruano
* Bayos:	Bayo Gordo (large grain)
	Bayo Rata (large grain)
	Garbancillo (medium size grain, small grain)
* Black	dull (small grain)
	shiny (medium size grain)
* Pintos	Flor de Mayo
	Pinto Americano
	Ojo de Cabra
	Cacahuate
* others	white seeded (small grain, medium size grain)
	red seeded (medium size grain, large grain)

That diversity has stimulated in Mexico an intensive selection work for the commercial materials whose color grain is different from black. In addition, varieties have been obtained through hybridization.

Bean research has been initiated in Mexico around 1936 when the first collections of Mexican materials were made. In 1949, in Tlalnepantla, Mexico, the first crosses were obtained between canario bean and bayo bean, when [researchers] were looking for bush types and types III that were resistant to rust, anthracnose, and bacterial blight. In 1949, the varieties "Rocamex 1", "Rocamex 2" and "Rocamex 3" selected by Wellhausen and others were released. Later on, these varieties were known as "Amarillo 154", "Negro 150", and "Bayo 158", respectively.

In 1955 seed of the variety "Canario 101" were distributed, resulting from individual selections in a collection found in Tacámbaro, Michoacán. Together with this variety the varieties "Bayo 158", "Bayo 160", "Bayo 161", "Negro 150", "Negro 151", and "Pinto 162", as a result of individual selections done in 1953. The first varieties obtained from crossing [activities], "Canocel", "Bayomex", and "Negro Mecentral", were released in 1959.

In 1958, in the Experimental Station of Cotaxtla, Veracruz, three new varieties of black bean were released: "Antigua", a selection of Guatemalan [material], "Actopán", and "Jamapa". The later has been one of the most famous bean varieties because of its wide stability, that has kept over more than 20 years. It has been developed by F. Cárdenas from a selection of 15 lines in the collection "Veracruz 86", in Paso de Ovejas, Veracruz, in 1955.

At the beginning of the 1970s, the Center of Agricultural Research of Sinaloa (now CIAPAN)

started with the distribution of new bean varieties developed in this center by the team led by Héctor López. Few varieties such as "Azufrado 33" were obtained through individual selections, while other [varieties] such as "Canario 78" (Ahome), "Azufrado 100" (Cahita 100), "Culiacán 200", "Azufrado Pimono 78" (Mayocoba), "Canario 72"(CIAS 72), and "Toche 400" [were obtained] through hybridization

In the center-northern [part] of the country the following varieties "Pinto Nacional 73" (Pinamerpa), "Bayo Calera", "Delicias 71", "Bayo Baranda", "Bayo Durango", "Pinto Fresnillo", and "Ojo de Cabra 73" (Ciechi) have been produced. In the state of Tamaulipas, the varieties "Agramejo", "Azabache", "Mulato", and "Ciateño" were obtained.

Only three names of persons have been mentioned in this review of varieties, in order to mention three steps got in bean breeding in Mexico: the one corresponding to improved varieties up to 1959, the appearance of "Jamapa", one of the varieties with largest diffusion in the world, and a third step including the work of Héctor López, who died untimely in 1978, and who participated actively in varietal development in the state of Sinaloa.

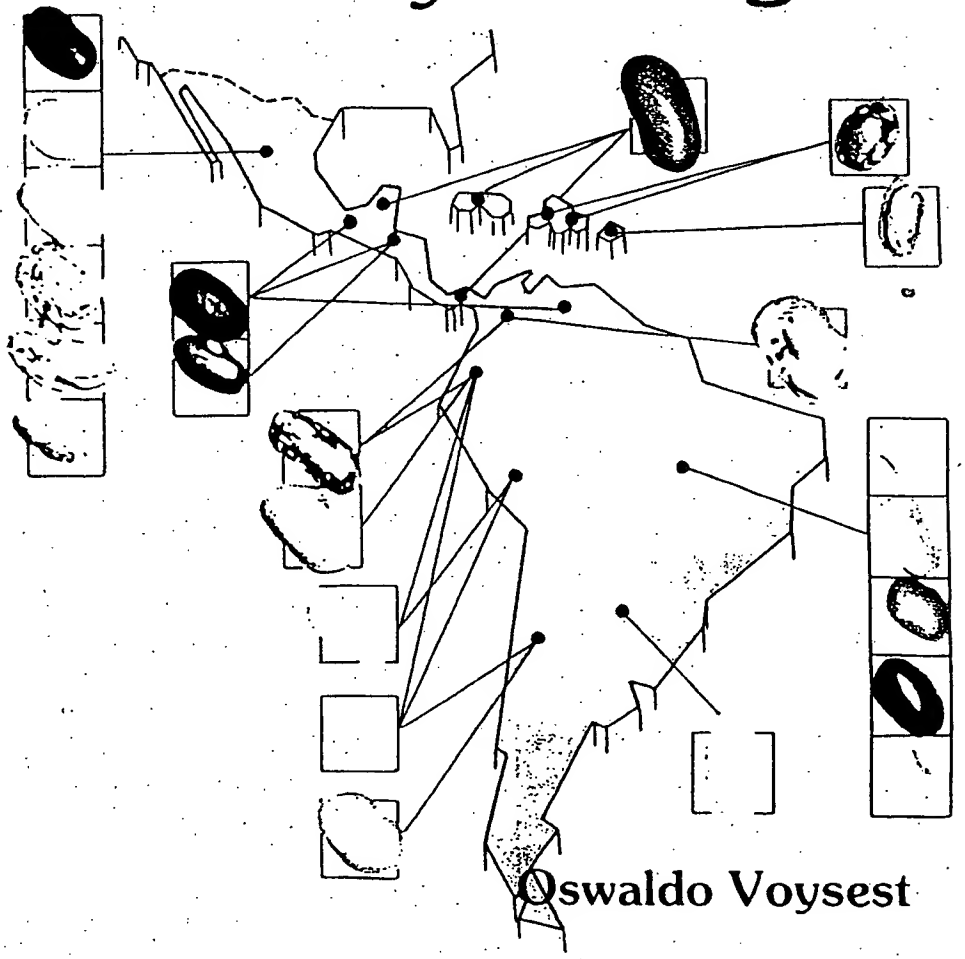
Given the large number of varieties produced in Mexico and the way its bean programme is organized - coordinated by disciplines at natioinal level , it is difficult to give credit to bean breeders for the creation of [certain] varieties without seriously missing out [names]. About 60 professionals of the Bean Programme of the National Institute of Agricultural Research (INIA) carry out research activities in 29 experimental stations. Genetic improvement work is distributed among five well defined ecological zones. In each of them one experimental station is carrying out the bredding programme, and is responsible to generate the materials that shall be distributed to other experimental stations in its ecological area, where these materials will be tested by multidisciplinary teams.

In Box 19 the main varieties grown in Mexico are presented.

ISBN 84-89206-26-0

CIAT
Centro Internacional de Agricultura Tropical
Apartado Aéreo 6713
Cali, Colombia

Variedades de frijol en América Latina y su origen



Oswaldo Voysest

CIAT
Centro Internacional de Agricultura Tropical